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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues from 235 donors (200 women, 35 men). In order to Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. The study of carcinogen-DNA adducts can provide corroborative evidence for the importance of genetic susceptibilities in breast cancer risk. We are establishing new assays for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies, and determine adduct levels in relation to metabolizing gene polymorphisms. The originally proposed assay is novel because one uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). We are now also using an enzymatic postlabeling method that will still rely upon ^{14}C AMS detection, but is specific for 4-aminobiphenyl. We are also developing a capillary HPLC and laser-induced fluorescence for polycyclic aromatic hydrocarbons. Once validated, we will learn the variability for understand the determinants of DNA adduct formation in the breast tissue, we also have been identifying cytochrome P450 immunostaining in the same tissues. Concurrently, we have been collecting and using cultured breast strains from normal donors to determine in vitro adduct formation levels and correlate these levels with p53 induction, hypermethylation of promoter regions in tumor suppressor genes, and mitochondrial mutations. Thus, this study will provide new information about genotype-phenotype relationships.

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Table of Contents

(4) Introduction

Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Currently, many ongoing breast cancer studies are exploring risks related to genetic polymorphisms in these genes. Yet these studies by themselves do not provide absolute proof of etiology or causality. Thus, corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. We are focusing on carcinogen-DNA adducts because they are promutagenic and lead to alterations in cancer susceptibility genes. They also serve as a marker of the biologically effective dose of a carcinogen, indicating a person's phenotype for metabolism, DNA repair and apoptosis. Several available carcinogen DNA-adduct assays might be useful here, but they are not sufficiently specific and/or sensitive for testing mechanistic hypotheses in humans. We are attempting to establish a new assay for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies. The assay is novel because it uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). We will develop and rigorously validate the assays using benzo(a)pyrene- (BPDE) and 4-aminobiphenyl (4-ABP)-related adducts as prototypes. Other methods are being explored in the event that finalization of the proposed method is not possible or if other, less labor intensive methods, can be done. With the development of an assay, we will learn the variability for DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues, and explore relative levels in liver tissue from autopsy donors. From a subset, 30 matched blood samples will be used to determine the relationship of breast levels (i.e., the target organ) to blood (i.e., the surrogate tissue). Finally, in these subjects, we will perform assays for genetic polymorphisms, to assess the association of "at risk" genetic variants with higher breast adduct levels. We also will test genotype-phenotype relationships by culturing primary breast cells from the same women, correlate adduct levels from in vitro carcinogen exposure and determine p53 response. This will allow us to establish the variability in the population for p53 induction from carcinogens, and might imply an independent risk for women with a low response.

During the interim between awarding the grant and receiving the funding, Dr. Shields decided to move his research laboratory from the intramural program of the National Cancer Institute to the Lombardi Cancer Center (LCC) of Georgetown University, effective January 1, 2000. Thus, it was decided to delay the implementation of the project until the budget and project design could be reorganized. First, it was inefficient to begin the project at NCI, which would last only three months and then require an interruption of the work and change in personnel. Upon arriving to the LCC, there was additional time needed to hire a postdoctoral fellow and establish the laboratory, although some work began almost immediately by Drs. Shields and Goldman. Thus, this project was not fulling implemented until about 6 months after the initial project date. All needed equipment, reagents and tissue samples were either transferred from the NCI, or purchased new at the LCC.

It should also be noted with the transfer of the grant from the NCI to LCC, there was a loss of available money because the NCI does not require overhead costs, while the LCC does. The DOD was requested to increase the cost of the total award to cover the additional overhead. This was denied and so there was a required rebudgeting to allow for the overhead, which reduced the available direct monies by almost 30%. Thus, some tasks may not be accomplished by the end of the three year grant period.

5) Body

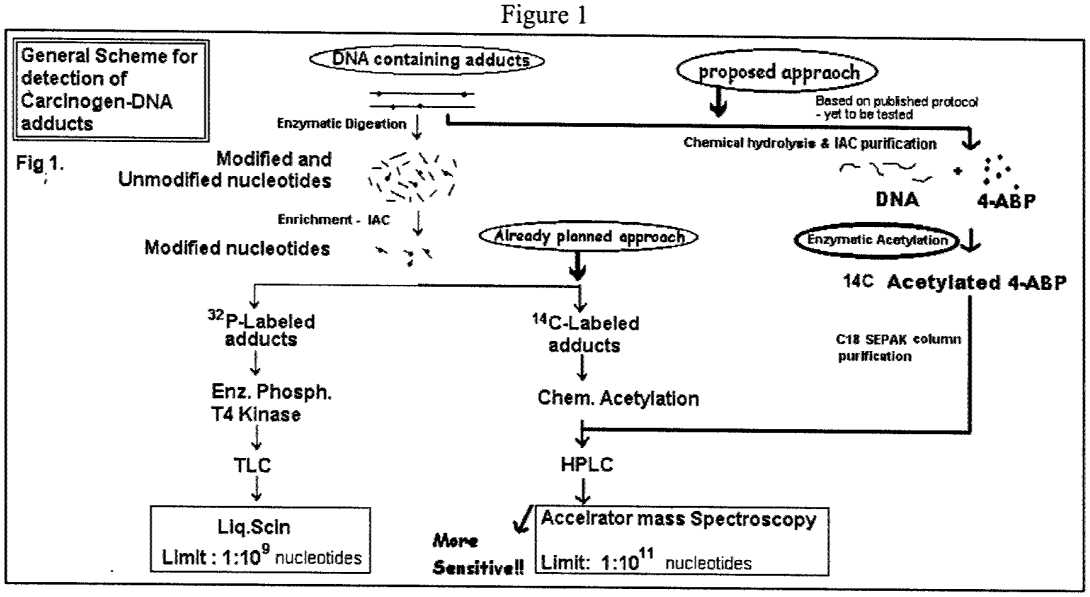
Task 1: **To develop a ^{14}C-postlabeling method with acetic anhydride using micropreparative techniques for chemical specificity and AMS for sensitivity (CAP). BPDE and 4APB adducts will be used as prototypes. (Months 1 -18)**

As will be described, we had been simultaneously developing the CAP method for BPDE and 4ABP. However, other opportunities have arisen, such that we now have been developing alternate detection methods for both BPDE and 4ABP. We will present our progress for CAP of both adducts and then a new enzymatic method for the detection of 4ABP using n-acetyltransferase (NAT) and acetyl-coA, and another new assay using capillary HPLC and laser induced fluorescence method (LIF) for the detection of BPDE adducts.

^{14}C Postlabelling method for detection of 4-ABP adducts

Figure 1 shows three possible methods for postlabeling 4-ABP adducts. On the left is the more traditional approach of using ^{32}P-postlabeling. On the right, is the newest method using NAT, and in the middle is the method proposed in the grant using acetic anhydride.

Figure 1



Chemical acetylation using acetic anhydride for 4ABP detection

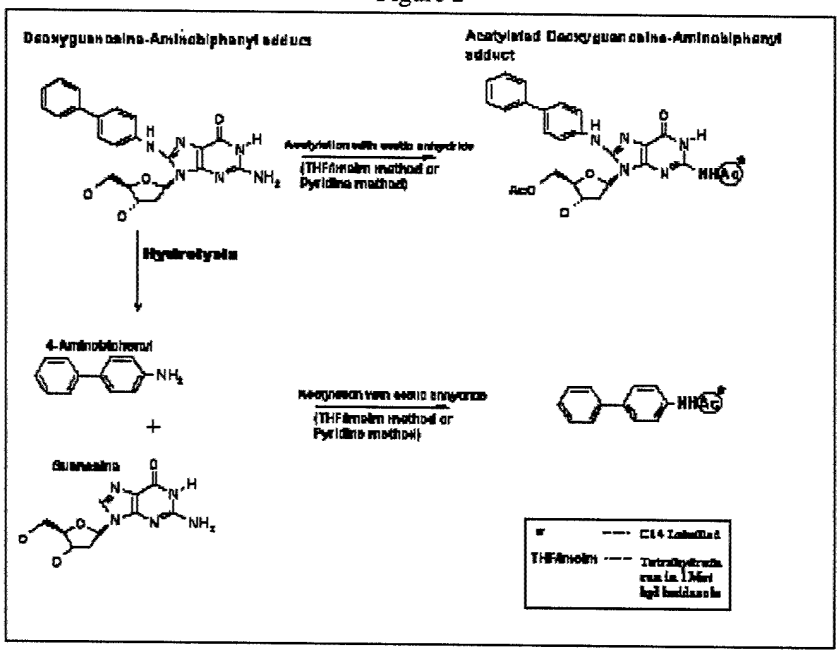
Figure 2 describes the reaction scheme for chemical acetylation of 4-aminobiphenyl isolated from DNA adducts.

Standardizing the methodology involved the following important steps:

1. Normalize the acetylation procedure of the starting material (4-ABP/ABP-Gu adduct) to obtain maximal yields. Standardize the reaction conditions with respect to temperature, duration of reaction, and amount of acetic anhydride required for acetylation (reactions would be initially carried out using cold acetic anhydride). Use HPLC as means for resolving products and isolating adducts. Once the profiles the compounds have been set, scale down the amount of starting material (4-ABP) and the amount acetic anhydride. Then standardize using 14C labeled acetic anhydride.

2. For immunoaffinity chromatography (IAC) for adduct purification, validate the ability of 4-ABP to bind to antibodies generated against ABP-Gu adducts. (Antibodies have been obtained from both Gerald Wogan and Regina Santella to have suitable antibodies for

Figure 2



IAC.) Bind the antibodies to columns (obtained commercially). Calibrate the binding capacity of both 4-ABP and ABP-Gu using CapLC with appropriate controls. Should the binding not be possible or efficient enough, we will need to make antibodies against 4-ABP itself.

3. Prepare standards of ABP-Gu adduct by established protocols (from existing literature) and use this in validating binding efficiencies of antibodies.

4. Standardize the hydrolysis step of ABP-Gu.

5. Perform AMS.

The yields of acetylation were initially assessed with non-radioactive acetic anhydride. The yields were compared with 4-aminobiphenyl-guanosine as a standard using HPLC. The acetylation had been carried out using two different methods:

1. Acetic anhydride dissolved tetrahydrofuran containing 16% methyl imidazole
2. Acetic anhydride dissolved in pyridine.

The reaction protocol was:

1. 1:1 of the starting material (4ABP-Gu) was dried in vacuum for ~15-20 min (drying time was standardized and its effect on elution profile was also verified to be OK).
2. 500:1 if THF/MeIm was taken in a dry vial(20 :1 of anhydrous pyridine in the alternative method). 3:1 of anhydrous acetic anhydride was mixed with this and instantly vortexed. 30:1 (from the THF/MeIm mix) or the entire pyridine/Ac. The mix was then added to 4ABP-Gu (4-ABP alone).
3. The reaction was allowed to proceed for 20 mins (THF/1MeIm) [2hrs in case of pyridine method].
4. The reaction was terminated by adding 70 :1 of water. Subsequently, the samples were dried in vacuum for ½ hr. The samples were redissolved in 100% methanol, dried. The samples were dissolved 5-20% acetonitrile before HPLC analysis.

For the 4-ABP acetylation, we have thus far mostly focused on the pyridine based

method, while for the dG-8-ABP adduct acetylation both the methods (pyridine and THF/1MeIm) have been used. Elution profile of 4-aminobiphenyl, as characterized by the gradient profile, is given below using a C18 column; acetonitrile (5%) and water (95%).

Gradient profile in HPLC:	0-2 min	-	45% acetonitrile
	2-5 min	-	grad 45-75% acetonitrile
	5-9 min	-	75%
	9-11 min	-	grad 75-95%
	11-13 min	-	95%
	13-15 min	-	grad 95-45%
Run parameters: Flow rate		-	1ml/min
Resting backpressure		-	82-85 bars
Injection volume		-	10:1

With the above parameters, the 4-aminobiphenyl eluted out at 7.76 min. The absorption maxima was ~280 nm. A small related peak could be seen at 7.06 min. The ABP-Gu adduct eluted at ~3.5 min. The absorption maxima was ~300 nm.

The yields of acetylation were standardized for 4-ABP. In case of ABP-Gu adducts the yields were not efficient (<50% yield of the acetylated product – as measured by UV detection in HPLC). Since the yields were better in case of 4-ABP (~ same as starting material), the focus was shifted to 4-ABP acetylation.

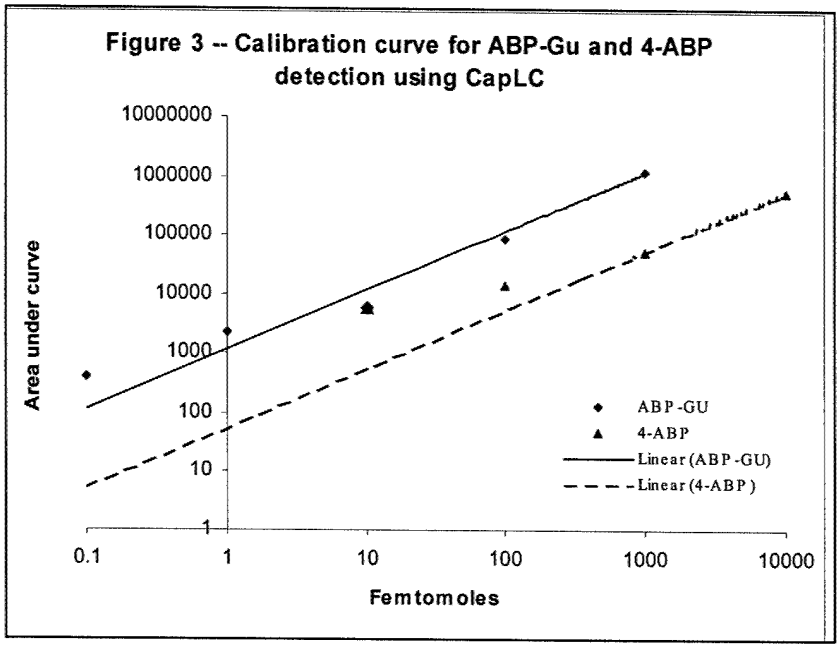
The optimal conditions to achieve acetylation of 4-ABP was observed at 37°C with an incubation time of 2 hrs. Mass spectroscopy was used to confirm the acetylated 4-aminobiphenyl and the control-unacetylated samples at the facility in Pharmacology department using an HP1100 GC MS instrument. Samples were injected directly into the MS for analysis based on run parameters standardized earlier for 4-ABP. The presence of the acetylated compound was inferred using the anticipated molecular weight. A near quantitative yield of the product from the starting material was observed and there were no other side products. The MS of 4-aminobiphenyl alone also showed the presence of a 212 molecular weight peak that corresponded to the acetylated product. Interestingly, the HPLC profile also showed a peak at the RT where the product elutes out.

The existing reaction protocol gave about 2000-fold excess of Ac2O over starting material (1 nanomole). This meant one vial of labeled acetic anhydride could successfully acetylate about 10 DNA samples. The amount of Ac2O was scaled down serially to observe the minimum requirement for acetylation. The yields still stood the same when the amount of Ac2O was diluted up to ten fold below the existing amount. This meant greater amount of samples could be acetylated for the same of labeled Ac2O. Since the amounts of 4-ABP are expected to much lower than the presently used concentration, more samples can be expected to processed if the amount Ac2O could be further scaled down. However, when the amount of starting material and the Ac2O amount were further scaled down, there were problems with yields. This problem is presently being addressed.

A calibration curve was established (Figure 3).

Antibodies to ABP-Gu adducts were obtained from Dr. Regina Santella at Columbia University in the form of ascites and were stored at -70?C. The antibodies were bound onto a column containing immobilized protein-G (commercially obtained kits) using the following procedure:

1. Load 10ml of ascites solution (~10mg of Ab) onto the column.
2. Equilibrate the column with 5 ml of Antibody Binding/washing buffer.
3. 10 ml of ascites solution + 10 ml of PBS buffer.
4. Load 2-4ml onto a column – gentle inversion – tap end of bottom.
5. Mix by gentle inversion for 30 minutes @ room temperature.. Remove top and bottom caps sequentially and allow passing out the remaining solution.
8. Repeat procedure 'c' and 'd' until all the ascites has been loaded onto the column



9. Wash extensively with Bind/wash buffer (4-5 times).
10. Cross Link the bound antibody with DSS
11. One vial content of DSS/1 ml of DMSO. Mix with 1.5 ml cross linking buffer (from kit).
12. Load onto column, mix and allow settling.
12. Wash with 1 x 5ml of cross-linking buffer.
14. Block the remaining active sites

15. Load 2ml of blocking buffer onto column, mix by inversion, allow settling the column and allow the solution to pass out.
16. Wash with elution buffer and allow flowing through (5 ml first time, 2ml next time). Store the column @ 4°C (with 0.02% NaN3)

Aliquots from this column were used load small columns that were used to check for binding of capacities. The following protocol was used to run the samples and process them for detection by CapLC electrophoresis (using UV detection).

1. Elute out the old solution containing NaN3. wash the column with 2-5ml of bind/wash buffer.
2. Take 20:1 of gel from the already prepared Immunoaffinity column into fresh smaller columns.
3. Wash with bind/wash buffer twice.
4. Load a known amount of ABP-Gu std (100 femtomole) from a diluted stock onto the column and collect the fractions.
5. Gently place the white frit on top of the column.
6. Mix by gentle inversion for 1 hr and allow to settle.
7. Dry down the sample and redissolve in water. Same as above but sample not passed through column.- to rule out non specific binding.
8. Do the same as in '6' for 4-aminobiphenyl.
9. Measure concentration of ABP-Gu and 4-ABP before loading. After loading and collecting, determine efficacy of generated antibodies.
10. Use appropriate starting material and determine background and recovery of CAP labeled adducts.

A calibration curve using tritiated label adducts was generated for detection of 4-ABP and ABP-Gu. 4-ABP with a suggested limit about 10 femtomoles/l (Figure 3). Using this, around 1 picomole was selected to monitor the binding capacities for IAC optimization. There have been problems presently carrying out this protocol. Binding of either ABP-Gu adducts or 4-aminobiphenyl could not be observed thus doubting the presence/binding efficiencies of the generated antibodies. The problem is being tackled by checking if antibodies have bound to the columns (assay the amount of Antibodies in ascites as well as columns using ready-made kits). Also, there has been a problem of drying out of the samples after they have been eluted out in methanol. Presence of small amounts fat like material is suspected. This is being tackled by using Tween-20 (<5%) and 20% methanol.

The CAP method is currently on hold, pending the development of the assays described below.

NAT Postlabeling of 4ABP

The proposed approach envisages the use of an enzyme N-acetyltransferase to incorporate the 14C in the released 4-aminobiphenyl (after hydrolysis from DNA). This is distinctly different from the 32P postlabelling approach, which also uses an enzyme. The specificity of the presently used enzyme to 4-aminobiphenyl (or any related arylamine) confers a unique advantage in detection of the adducts.

This technique differs from

1. The enzymatic approach used in 32P-postlabelling, in that the enzyme is targeted towards 4-aminobiphenyl alone rather than adduct. Hence the specificity for the enzyme on the substrate could remain relatively unaffected and be more amenable to enzymatic modification. The release of 4-aminobiphenyl from the DNA adduct would be achieved by simple alkaline hydrolysis based on an established protocol 1, thus retaining the quantity of the carcinogen from the sample as unaffected as possible.
2. Either of the earlier approaches (i.e. ^{32}P-postlabelling or chemical 14C-postlabeling) in that it does away with the necessity of enriching adducts in the sample, such as through IAC. Although, the IAC procedure can be used effectively in this approach too, should the actual specificity not be achieved by enzyme alone.
3. Chemical postlabelling in that it hopes to do away with extensive cleanup procedure to wash out the excess labeled 14C (also thereby preventing loss of sample)

Standardizing the methodology involves the following important steps.

1. Test the hydrolysis of 4-aminobiphenyl from prepared 4-aminobiphenyl-DNA adducts (based on a protocol from an established hydrolysis procedure above for CAP. This would demonstrate the successful release of 4-aminobiphenyl after the alkaline hydrolysis step.
2. N-hydroxy-4-aminobiphenyl is synthesized as a prelude to this procedure for preparing 4-ABP-DNA adducts. Once the hydrolysis step is established, the reaction would be advanced to use of tritiated N-hydroxy-4-aminobiphenyl. Synthesis would be by another established procedure.
3. Prepare 4-ABP-DNA standards using calf thymus DNA.
4. Carry out alkaline hydrolysis of the adducted DNA 1 and determine the efficiency of release from DNA.
5. Standardize the hydrolysis procedure of 4-aminobiphenyl from already synthesized ABP-Gu adducts.
6. Normalize the enzymatic acetylation procedure of the starting material, 4-ABP.
7. Standardize the reaction conditions with respect to temperature, duration of reaction, and amount of acetyl CoA required for acetylation (reactions would be initially carried out

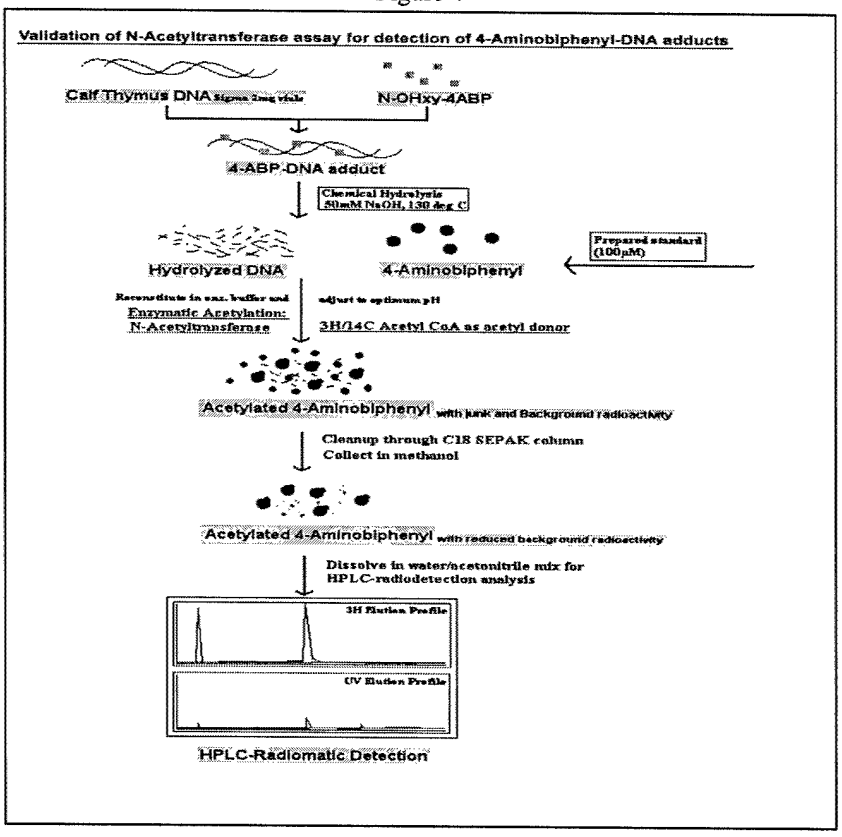
using cold acetyl CoA).

8. Test the efficiency of recovery after subjecting to C18 SEPAK cleanup.
9. Check the elution times of the unreacted Acetyl CoA with respect to the acetylated 4-ABP to ensure non-bleeding of the unreacted compound into the product peak.
10. Use HPLC initially as means for detection of products. Once the profiles of the compounds have been identified, scale down the amount of starting material (4-ABP) and the amount acetic anhydride. Subsequently standardize using 14C labeled acetyl CoA.
11. Validation of the enzymatic acetylation from prepared DNA-4-ABP adducts using 3H Acetyl-CoA. This step would demonstrate the viability of the proposed method. Prior to this procedure, the 4-ABP is hydrolyzed from DNA samples. Subsequently, the released 4-ABP could be either separated using hexane extraction or the entire hydrolysate could be subjected to an enzymatic reaction (preceded by adjustment of pH for optimal reaction conditions).

Following were the main results of the steps outlined above:

Preparation of the 4-ABP-DNA adducts: Given below in Figure 4 is the schematic by which the 4-ABP-DNA adduct standard was prepared.

Figure 4



Validation of hydrolysis procedure:

1. Preparation of 4-ABP-DNA standards was followed by an established procedure 2. Calf thymus DNA was used as the DNA source and N-hydroxy-4-aminobiphenyl was used to add it to DNA. Preparation of N-hydroxy-4-aminobiphenyl carried out by partial

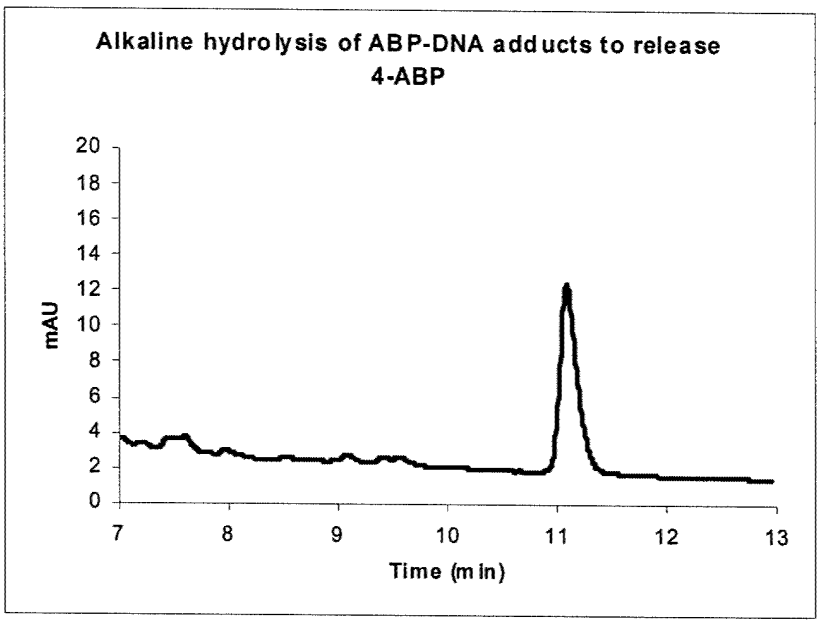
reduction of 4-Nitrobiphenyl 3. NMR spectra confirmed the synthesis of the compound. Since the compound was unstable it was kept stored under argon gas trap at -20°C - -80°C. Solutions of the compound were prepared in ethanol or methanol but they were used as soon upon preparation.

2. N-hydroxy-4-aminobiphenyl was added to DNA (already dissolved in a suitable solvent 2 so as to keep the ratio of the pre-carcinogen to DNA nucleotides at 1.8×10^{-2}). Though this was starting ratio, the final binding efficiency could be determined by release of 4-aminobiphenyl after successful hydrolysis. After an overnight incubation, unreacted N-hydroxy-4-aminobiphenyl was extracted into an equal volume of hexane twice over. DNA was then precipitated by the phenol:chloroform procedure, re-dissolved in water and was then re-estimated to check for DNA recovery. A near complete recovery was achieved.

3. The adducted DNA was then subjected to an alkaline treatment (50 mM NaOH) in a glass tube and kept overnight at 130°C 1. The released 4-aminobiphenyl was then extracted into Hexane (two times) and then dried in a rotary evaporator. After re-dissolving in 1 ml 20/80 Acetonitrile/water mix, the solution was then analyzed by HPLC to detect for the presence of 4-aminobiphenyl peak.

4. As anticipated, there was a peak at 11 min - by an already established gradient profile of water/Acetonitrile (Fig. 5) . This peak was well separated from that of the starting material (N-hydroxy-4-aminobiphenyl – Fig. 6 – see below), which eluted at 8 min. The peak had spectra corresponding to 4-aminobiphenyl (max – 280 nm). Fractions containing this peak were pooled and dried and concentrated to analyze by Mass spectroscopy. A molecular weight of 169 corresponding to that of 4-aminobiphenyl was

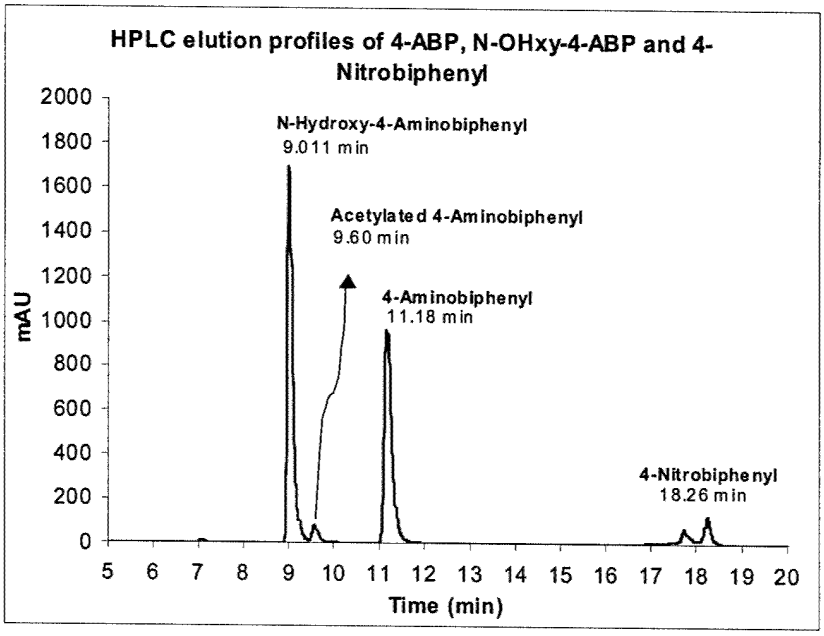
Figure 5



obtained thereby confirming the nature of the released compound.

Thus, demonstration of the release of 4-aminobiphenyl made it possible to proceed further into examining the enzymatic acetylation process.

Figure 6



For the normalization of the enzymatic acetylation reaction, the reactions initially were standardized using cold acetyl CoA and 4-aminobiphenyl standards. Limits of detection were also tested using HPLC coupled to radiomatic detection of labeled standards.

Cold acetylation (without the use of 3H label)

1) The enzymatic assay was performed using the enzyme N-acetyltransferase (E.C. 2.3.1.5) and Acetyl-CoA as the carbon donor. Unit definition of the enzyme was as defined by the supplier. The assay procedure was modified from the protocol provided by the commercial supplier (Sigma Inc). A typical reaction setup is described below

Reagent A (100 mM Potassium phosphate buffer) – 60µl
Reagent B (100 mM ß-mercaptoethanol) – 15µl
Reagent C (30 mM EDTA) – 10µl
Reagent D (4-aminobiphenyl) – 10µl (100µM – i.e. 1 nanomole of substrate)
Reagent F (Enzyme solution in buffer – 100µl

Mix well and split into two aliquots of 97.5 µl each

Reagent E (Acetyl CoA) – 2.5µl (2.5µl water, if it is a blank)

The reaction mix was incubated at room temperature for 20 min – 60 mins.

2) Following this the reaction mix was subjected to a cleanup using a SEPAK C18 column. The procedure was as follows

a. Place the columns (one for a each reaction) over a vacuum manifold that are accordingly connected to the tube outlets inside the manifold.
b. Wash once using methanol (~ 1-2 ml)
c. Wash 3-4 times with water (~ 5 ml)
d. Load the column with the reaction mix. Suck the contents into trash (already placed

inside the manifold).

e. Wash again with water 3-4 times

f. Remove the trash container from within the manifold and replace with adjustable tube stand and place HPLC glass vials below each column.

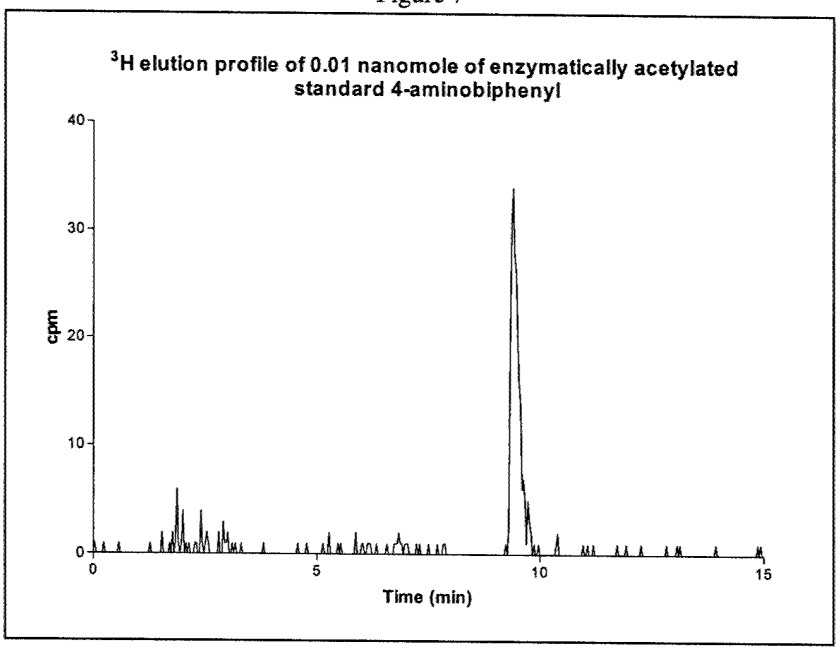
g. Load the column with a known amount of methanol and suck into tubes. Repeat the procedure 2-3 times with the total collected volume of methanol not exceeding 500-700µl.

h. Dry the collected volume using speedvac rotor (if it is a reaction mix containing acetylated 4-aminobiphenyl or else dry using gentle air flow method if the collected volume is from a Blank sample (4-aminobiphenyl is unstable when subjected to drying by speedvac in glass vial.

i. Redissolve in 100µl of HPLC run compatible solvent (20:80 ACN/H2O mix) and analyze using HPLC (by the already established gradient profile).

3) In the reaction mix containing the acetylated product, there was an expected product peak at 9.5 min with a concomitant disappearance of the 11.0 min reactant peak (Fig 7). The reaction system containing the peak gave out unreacted 4-aminobiphenyl (11.0 min peak). Parallel controls using enzyme blank (no enzyme in reaction) were also carried out to rule out

Figure 7



non-enzymatic conversion to end product.

4) The detection limit using HPLC coupled to radiomatic detection was 0.01 nanomole of standard 4-aminobiphenyl.

5) Elution profile of acetyl-CoA alone showed the compound to be eluted out in the solvent front itself, thus resolving the separation of the reacted and unreacted peaks.

3H labeled acetylation

1) The procedure was similar as described above with the exception that 1 nanomole of the standard 4-ABP was replaced by either the hexane extract of the hydrolysate (dried and re-dissolved in methanol) or the hydrolysate itself (pH readjusted to 7.4 for optimal reaction conditions).

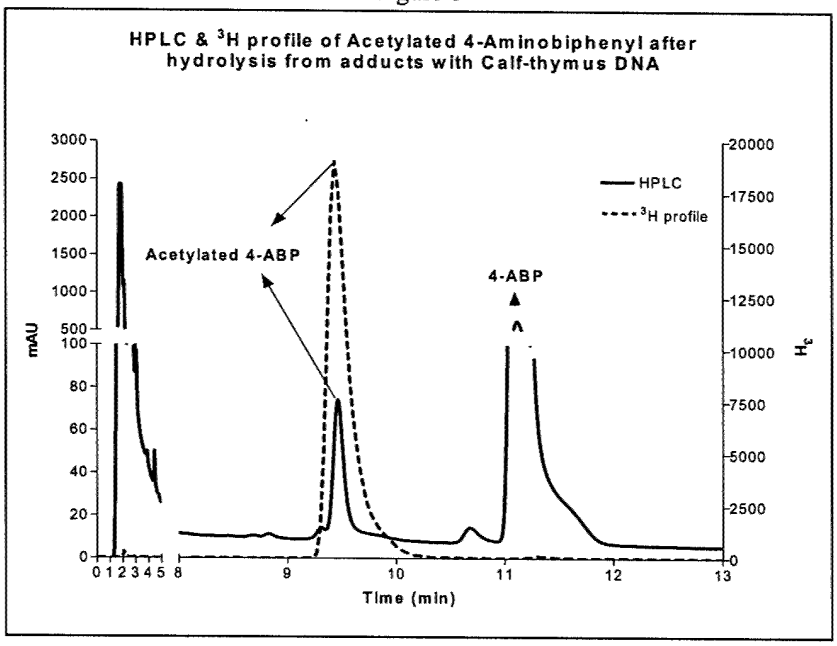
Hydrolysis procedure resulted in drying up of the reaction mix which was followed by loss of yield of the released 4-ABP for adducts. To prevent this, the reaction was carried out in 5ml amber colored ampules and heat-sealed.

Once the recovery was standardized, enzymatic reaction was carried out by breaking open the seals. pH was readjusted in the vials for optimal enzymatic reaction conditions. Reaction protocol was adapted from already standardized procedure.

The enzyme was successful in acetylating the released 4-aminobiphenyl from adducts. This was evidenced by the appearance of the product peak at 9.55 min (Figure 8). However, there was still a significant amount of unreacted 4-aminobiphenyl left. Reaction conditions need to be further standardized like, heating drying, pH adjustment. It is also possible some hydrolysis products cause reduction in enzyme activity.

Recovery of the acetylated products after SEPAK cleanup was also tested. Acetylated 4-aminobiphenyl was prepared and HPLC purified. SEPAK recovery was tested by passing the products through the column (as described above). Yield was compared that of the pure

Figure 8



acetylated 4-aminobiphenyl. There was a greater than 85±5 % recovery of the product.

2) When reaction was carried out with unhydrolysed DNA-4ABP adducts, the enzyme N-acetyltransferase was unable to catalyze the transfer of acetyl CoA in 4-ABP. Thus the reaction could be carried out only on hydrolyzed 4-aminobiphenyl.

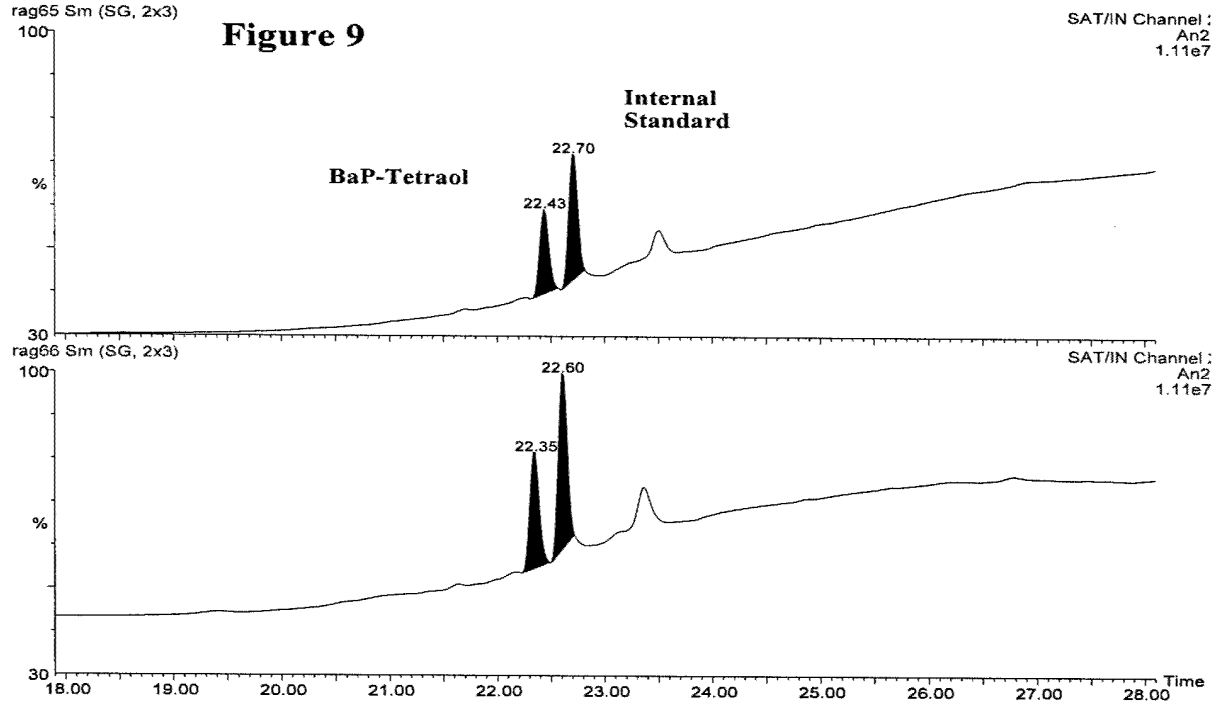
3) Adducts of 4-ABP-DNA have been prepared in varying adduct ratios. These will be serially diluted with unlabeled DNA to prepare varying levels of adducted DNA and these will be used to test the detection limits.

Future perspectives and plans:

1) Optimize hydrolysis efficiency using labeled acetyl CoA.
2) Standardize conditions for 14C reaction in lab; these include background survey checks of 14C. This is the most important part of the assay as Accelerator Mass Spectroscopy is an ultra-sensitive instrument to detect trace 14C in samples.
3) Carry out reactions with 14C acetyl CoA, test detection limits using HPLC and radiomatic detection. Carry out acetylation on serially diluted fractions of prepared 4-ABP-DNA adducts and count 14C in the HPLC fractions.

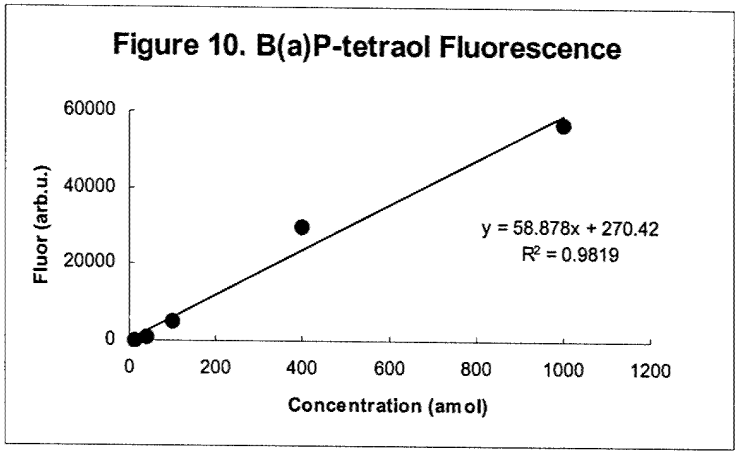
Adduct detection using capillary liquid chromatography with laser induced fluorescence (capLC-LIF)

One of the best methods for B(a)P quantification is the fluorescent detection of B(a)P-tetraol following HPLC separation of the acid hydrolyzed B(a)P adduct. The B(a)P tetraol has



strong fluorescence (λex=345 nm; λem=390 nm) which allows direct detection of the compound without labeling. We are taking advantage of this native fluorescence and further modifying the method to improve the detection sensitivity. To this purpose, we have recently installed a capillary LC system (Waters Inc., Milford, MA) together with a laser-induced fluorescence detector (Picometrics, Ramonville St Agne, France). The capLC decreases typical HPLC flow rates 1000 fold (from 1ml/min to 1µl/min). This promises an approximately 100 fold improvement in sensitivity over the HPLC/fluorescence when combined with the laser induced fluorescence detection (which can be focused on the capillary).

Both the capLC and the fluorescence detector are new instruments that have become available in the last few years. During the grant period, though, both instrument had technical problems that delayed somewhat our progress. The capLC had faulty valves causing inconsistent retention times. The Waters, Inc. replaced all three pumps on our capLC system. Since there are only few units produced each year and the demand for the instrument increases, the pumps were on back order for over six month. We have extensively validated the performance of the new



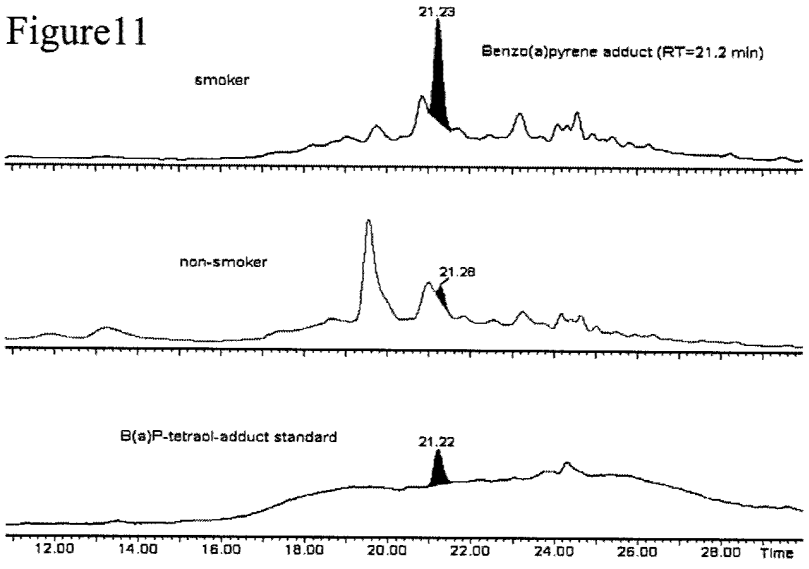
pumps since the installation in June 2001. We have shown that the retention time is now reproducible within 0.1% which is consistent with the manufacturer specifications **(Figure 9)**. This allows identification of the B(a)P by coelution at the retention time of a known standard.

The LIF detector was also damaged due to laser tube leakage. The laser head was repaired by Melles-Griot, Inc. (Carlsbad, CA). The repaired unit arrived in October 2001. We did verify that the sensitivity of the instrument was not affected. As the standard curve illustrates, we are still able to detect the B(a)P tetraol with linearity over several orders of sensitivity down to 20 attomol of the standard **(Figure 10)**.

To improve the quantification of the B(a)P tetraol derived from human samples, we have incorporated an internal standard into the chromatographic procedures for adduct quantification **(Figure 7)**. A number of standards were tested and the best standard was selected based on its fluorescence excitable at the 325 nm and its retention time under reverse phase chromatographic conditions. We have chosen a stereoisomer of B(a)P-tetraol which does not form *in vivo* yet has properties similar to the B(a)P-tetraol (rttc). The retention time of the internal standard (22.7 minutes) is close to the retention time of the B(a)P-tetraol (22.4 minutes) yet is baseline separated from the analyte. The addition of the standard allows normalization of the adduct levels and improves quantification.

Two methods for sample cleanup prior to analysis were compared: 1. On-line trapping of analyte on a precolumn with flow switching; and 2. Trapping of analytes on a seppak C18 cartridge. The on-line trapping requires use of the 3rd pump of the capillary LC system. This

Figure11



configuration increases the flow path and introduces unwanted variability in the retention time and broadening of the peaks. We have therefore focused on trapping of the B(a)P-tetraol on a C18 seppak cartridge (Alltech, State College, PA). The seppak is washed with 5ml of distilled water and the retained hydrophobic compounds including the B(a)P tetraol are eluted in 1ml of 100% acetonitrile and dried. The analytes are dissolved in 20% aqueous acetonitrile and separated by capillary LC using a 0.3x100 mm C18 column (Keystone Scientific, Bellefonte, PA).

We initially verified the applicability of the method to human samples. We have analyzed two human samples with B(a)P adducts previously measured by 32P-postlabeling. One sample was lung tissue of a smoker with high B(a)P adduct level by 32P-postlabeling, the second lung sample was a nonsmoker with B(a)P adduct below the detection limit of the 32P-postlabeling method. 0.05 mg of each DNA was hydrolyzed by 0.1N HCl for 4 hours at 90^{0}C. The DNA lysate was applied to a C18 seppak cartridge (Alltech, State College, PA) and washed with 5ml of distilled water. The retained hydrophobic compounds including the B(a)P tetraol were eluted in 1ml of 100% acetonitrile and dried. The sample was reconstituted in 20μl of 25% aqueous methanol and injected into the capLC using an autosampler. The analytes were separated on a β-basic 100 x 0.3 mm column (Keystone Scientific, Bellefonte, PA) using a linear gradient of water and acetonitrile from 5% to 50% acetonitrile over 45 minutes. The B(a)P tetraol was identified by retention time corresponding to the retention time of an authentic B(a)P standard (Chemsyn, Lenexa, KS). High adduct level by 32P-postlabeling was clearly higher by fluorescence (Figure 11). The sample undetectable by 32P-postlabeling was still measurable.

This initial encouraging result was however hard to reproduce. Repeated analysis of the sample did not show consistent peak pattern as noise from fluorescent impurities masked the signal. Variable amount of noise was recovered from the cleanup procedure and substantial modification of the procedure was necessary to improve the result. First, we did extensively clean (speedvac, HPLC autosampler, H2O and other solvents) or replace (vacuum manifold, collection vials, precolumn and filters) many components of the analytical path. Improvement in noise reduction was tested after these steps. We did find out that some impurity was removed by replacement of the precolumn and filters and by cleanup of the speedvac system. Some fluorescent impurities are always recovered from the HCl used for hydrolysis and from dH2O. This is true even though we use HCl extracted with organic solvents and highest purity HPLC grade distilled water. The fluorescent impurities from the HPLC grade H2O are especially striking when retrieved following concentration on the C18 seppak cartridge. The biggest source of impurities is in fact the concentration of impurities from H2O and DNA hydrolysis on the 50mg C18 seppak cartridge. The cartridge retains efficiently fluorescent impurities that completely mask the signal. When we attempted to construct a standard curve based on fluorescence of acid-hydrolyzed calf thymus DNA modified with 3H-BPDE, we were able to detect only >20 femtomol of the adduct (compared to 20 attomol of the purified standard). This is not acceptable for the analysis of human samples.

To alleviate this problem, we did start minimizing the procedure. At first we used a home-made C18 cartridge with ~5mg of C18 resin. This was prepared by aliquoting C18 suspension in isopropyl alcohol into a disposable 1ml polypropylene syringe with frits. The C18 was packed by flowing methanol through the cartridge under vacuum. This smaller cartridge decreased the noise but the recovery of analytes was variable likely due to the improper packing of the resin. Later on two products came to the market that helped substantially with the cleanup. Waters introduced the OASIS 96 well plate with 5mg resin per well (Waters, Milford, MA), and Whatman released a 96 well polypropylene bioplate SPE with C18-impregnated glass fiber (Whatman, Clifton, NJ). Both have sufficient quantities of resin to retain the analytes, but retention of the impurities is substantially reduced. The best results were obtained with the C18-impregnated glass fiber (Whatman, Clifton, NJ), which contains approximately 3mg of C18 per well. The polymeric OASIS resin from (Waters, Milford, MA) has somewhat higher fluorescent background. Our analyte recovery from the C18 impregnated glass fibers is consistently greater than 80% and we are able to quantify 500 attomol of B(a)P tetraol spiked into 50?g of HCL hydrolyzed DNA (compare to >20 femtomol detected under previous

conditions). The C18 cartridge is washed with 5ml methanol, equilibrated with 1ml distilled water, sample is loaded, washed with 2ml dH2O, the retained hydrophobic compounds including the B(a)P tetraol are eluted in 250?l of 100% acetonitrile and dried on a speedvac.

Another improvement of the protocol comes from introduction of a new analytical column. We started using an Aquasil C18 column (Keystone Scientific, Bellefonte, PA). This column retains some hydrophilic properties and improves separation of impurities from the analyte signal compared to a standard C18 column. The analytes are dissolved in 25% aqueous acetonitrile following speedvac drying of the hydrolyzed DNA/analytes purified on the C18-impregnated glass fiber (). The analytes are subsequently separated by capillary LC using a 5?m particle size, 0.3x100 mm C18 column (Keystone Scientific, Bellefonte, PA) using an isocratic elution at 35% aqueous CH3CN. The retention time of the analyte B(a)P tetraol rttc under these conditions is 8.4 minutes and the internal standard B(a)P tetraol rtct is baseline separated with retention time of 9.3 min. This improves not only the sensitivity (500 attomol of the standard detected) but also the analysis-throughput (10 minutesper analysis compared to previous 45 minutes). At present we are optimizing wash conditions on this 96 well cartridge and begin with construction of a standard curve based on 3H-BPDE modified DNA. We will attempt quantification of B(a)P adduct in human samples following validation of the procedure.

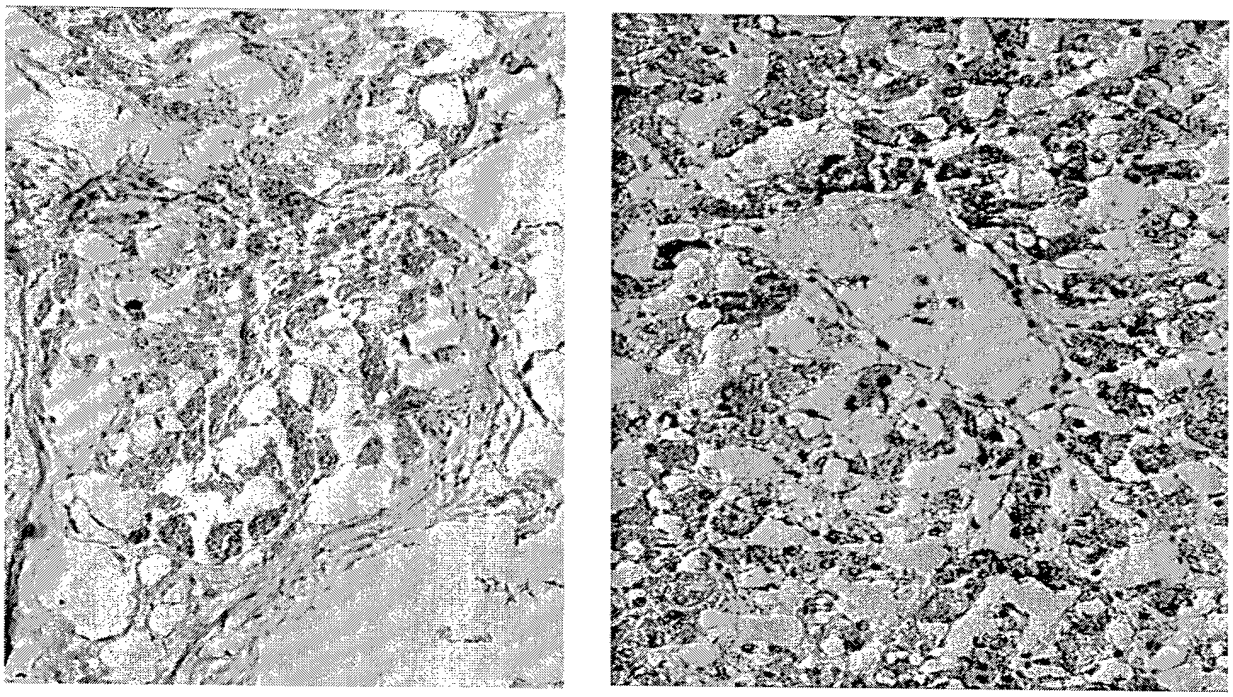
Task 2: **To demonstrate the use of the new adduct detection method in human breast tissues, livers, and blood from autopsy and surgical reduction mammoplasty donors. (Months 18 - 36)**

Autopsy samples as described in the grant proposal, collected at the NCI, are now at LCC. We have samples from matched breast, liver and lung samples. We also have samples from subjects who underwent reduction mammoplasty, and have developed primary strains from these same women. At the LCC, we have established the same study, where we recruit women who are undergoing reduction mammoplasty (about 1per week), have them provide a 2 hour long personal interview, and provide us with breast tissue and blood. We then culture the tissue as well as bank fresh frozen tissue. Thus far, 54 subjects have been recruited.

We have extracted DNA from the autopsy breast and reduction mammoplasty samples. The samples yielded at least 0.6 mg of DNA per gram of tissue. This quantity is in excess of our need to carry out the B(a)P adduct measurements (25ug per measurement). The excess DNA can be used for future expansion of the project to detect other adducts and related polymorphisms. We have purified the DNA by extraction with ethyl acetate and n-butanol (each in duplicate) to remove any unbound fluorescent impurities. These samples are ready for the hydrolysis and quantification of the B(a)P adduct.

Imunohistochemical Quantification of CYP1A1, CYP1B1, CYP3A

To better understand the carcinogenic pathway in smoking related lung and breast cancers, we are analyzing human autopsy tissues of smokers and non-smokers for parent polycyclic aromatic hydrocarbons, including benzo(a)pyrene, metabolic enzymes involved in activation of benzo(a)pyrene to the mutagenic benzo(a)pyrene diol epoxide, and the procarcinogenic B(a)P-DNA adducts. We have measured expression of the three major B(a)P activating enzymes, CYP1A1, CYP1B1, CYP3A in lung, breast, and liver tissues. This data will provide novel information and allow us to understand the mechanisms for DNA adduct formation.



CYP1B1 BREAST CYP3A LIVER

FIGURE 12

So far we analyzed 17 breast and 25 liver samples and there are 15 matched lung/breast pairs in the set (Figure 12). The expression is summarized in **Table 1**. we are also analyzing lung tissue so that we can compare and contrast results of an organ that clearly is susceptible to tobacco carcinogenesis. Most of the lung tissues express CYP1B1 and CYP3A, and 38 of 50 tissues express CYP1A1.

Future research will evaluate racial and gender differences in the CYP expression and induction by smoking, correlation of parent PAH compounds with expression of the metabolic

TABLE 1

	Lung	Breast	Liver
CYP1A1	38/50	13/16	0/27
CYP1B1	45/50	14/16	3/27
CYP3A	44/50	8/16	26/27

enzymes, and correlation of DNA adducts of benzo(a)pyrene with the CYP enzyme expression.

Task 3: **To use a novel breast primary epithelial culture system will be used to demonstrate the variability of p53 in the population. To determine p53 response in 40 human primary breast epithelial cells following exposure to benzo(a)pyrene (Months 30-36)**

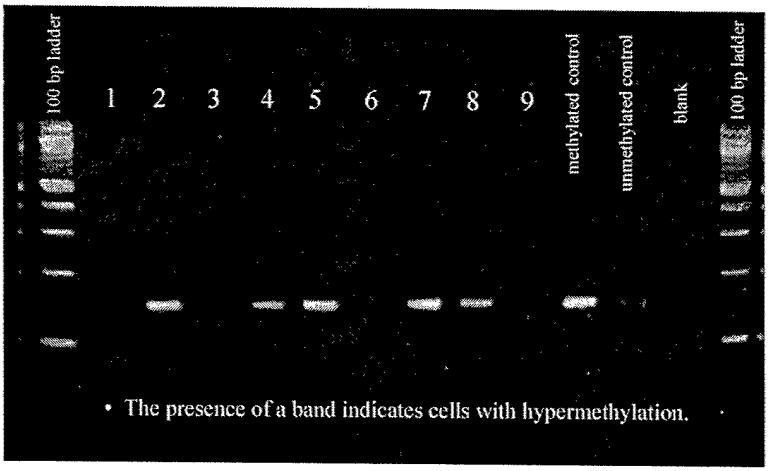
We have transferred 66 HMEC strains from the NCI. There is now an ongoing collection of tissues, with routine culturing at LCC (about 2 per month) –54 strains have been established. p53 data in response to irradiation has been tested in 11 of those NCI strains and 9 of the LCC strains. The NCI strains were used initially to develop the protocol for p53 protein measurement, and once that was established, data was collected on strains that were subsequently unfrozen. Many of the experimental problems have been worked out, i.e. the best way to culture the frozen strains for p53 experiments, the amount of tissue I need from LCC patients to establish a strain. The main problem that still exists with the NCI frozen strains is that many did not survive the freezing process, however, since we have multiple tubes for each strain, we are hoping that at least one tube is viable, and we will be able to get data for each strain. If this fails, there is sufficient accrual at LCC, with fresh tissue, that the total number of subjects is not problem.

At LCC, tissue is collected from disease-free women undergoing reduction mammoplasty within 4 hrs. following surgery. The tissue is digested with a collagenase/hyaluronidase mixture into organoids. The organoids are plated in flasks following a 24 hour digestion period. The flasks are maintained in serum-free mammary epithelial cell growth medium and fed three times a week. Epithelial cells are passaged at confluency, which they typically reach 3-4 weeks following digestion. Frozen strains are thawed and maintained in the same manner. Experiments to measure p53 are done at the second or third passage. The cells are plated in equal amounts in 35 mm dishes at about 70% confluency (usually about 1 X 10^5 cells/per dish). Cells are then exposed to gamma-irradiation in a JL Sheperd Mark I irradiator at doses of 5, 10 or 20 Gy. Controls are sham-irradiated cells.

Four hours following irradiation, proteins are extracted from the cells and used in western analysis. p53 is measured using the DO(1) antibody according to standard protocols. Beta-actin protein levels are also measured. The results are quantitated using densitometry and the p53 protein levels are normalized to the beta-actin protein levels to obtain the level of p53 induction. In some experiments, 4-aminobiphenyl (4-ABP) was applied to cells cultures. A cytotoxicity assay was used to determine the appropriate concentration of 4-ABP and the cells were incubated in 0.3 uM, 30 uM (the ED_{10}), or 3mM 4-ABP. Non-treated cells were used for controls. P53 and beta-actin proteins were measured as above.

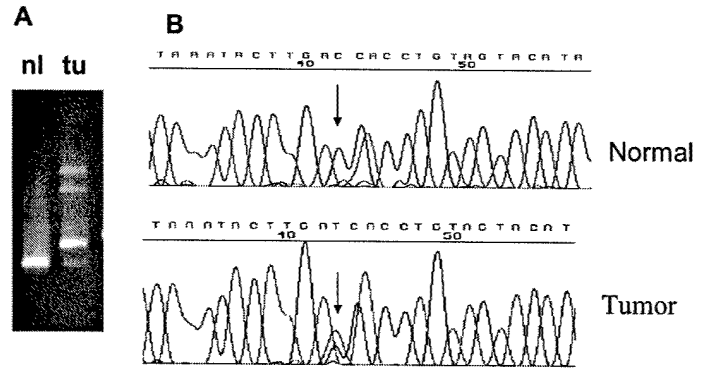
DNA damage resulting from chemical carcinogens and ionizing radiation has been shown to induce p53 in several experimental models. However, to our knowledge this has not yet been demonstrated in normal human mammary epithelial cells (HMECs). We have performed primary cultures of HMECs from 12 different cancer-free women undergoing reduction mammoplasties. The cells were passaged at confluency and experiments were performed at the third passage. The environmental carcinogen 4-aminobiphenyl (4-ABP) was prepared as a 10-fold dilution series with concentrations ranging from 30 mM to 30 nM and applied to the HMECs. Following a 24-hour incubation period, cell death was measured using a fluorescence assay. There was an increase of cell death at all concentrations of 4-ABP as compared to non-treated controls with a maximum two-fold increase in death with 30 mM 4-ABP. Using Western analysis, we have also shown an approximate two-fold increase in p53 protein in the HMECs of the one strain tested thus far with 0.3 uM 4-ABP. Additionally, we investigated the effect of γ-irradiation on the six HMEC cell strains. The HMECs were each exposed to γ-irradiation at doses of 5, 10, and 20 Gy. p53 protein was detectable by Western analysis at all levels of irradiation, and at baseline in non-irradiated controls. An approximate 2-fold increase was demonstrated at the highest level of irradiation (20 Gy) from control levels (0 Gy) in all six strains. However, there was a differential amount of p53 induction among the strains at lower doses of γ-irradiation (5 and 10 Gy). The six strains can be separated into three distinct groups for response (none, low, and high) with three strains showing no significant increase in p53 at 5

Figure 13
P16 Methylation in Normal Human Breast Tissue



and 10 Gy; two strains demonstrating a dose-dependent increase in p53 at 5 and 10 Gy; and one strain showing an initial 2-fold increase in p53 at 5 Gy and then leveling off at 10 and 20 Gy. We observed a differential amount of p53 induction among the strains at different doses of gamma radiation. Based on our results the strains can be separated into 3 groups: low, medium and high responders. These results indicate that the p53 response to DNA damaging agents differs in the breast and we hypothesize that such differences might influence breast cancer risk.

Figure 14
Mitochondrial Mutations in Breast Cancer



Panel A: TTGE detection of heteroplasmic mutations in a breast tumor case.
nl, normal; tu, tumor.
Panel B: Sequence analysis revealed a heteroplasmic C16147T mutation (arrow).

In order to ensure that the variability that we have observed was present in epithelial cells and that the differences were not due to differences in epithelial to fibroblast rations, we used cytokeratin staining by immunofluorescence to determine the percentage of fibroblasts

contamination in human mammary epithelial cell cultures. The cultures expressed 80-99% epithelial cells after cytokeratin staining by immunofluorescence.

In 2002, we presented to the AACR annual meeting data for senescence-associated beta-galactosidase, performed for cells in passage and cytokeratin staining. The latter was done by immmunofluorescence to determine the percent of epithelial cell culture contamination by fibroblasts. Cytokeratin staining showed that the cultures expressed 80-99% epithelial cells. Then, cells were irradiated at 5Gy, 10Gy and 20Gy in order to generate a dose response curve for incorporation of bromodeoxyuridine (BrdU), to be used to indicate the proliferative index. The BrdU incorporation assay showed that even at the lowest dosage (5 Gy) most of the cells arrested in G1/S phase.

Separately, we assessed whether we can observe estrogen receptors in normal breast tissue. Using 6 frozen dissected tissues, we performed Western blots. We observed the ER alfa and beta expression in 5 of 6 samples for the tissue collected from reduction mammoplasty patients. We then used these tissues to assess for hypermethylation of p16 and BRCA1. In these normal women who never had cancer, we found evidence for both (Figure 13). Also, we found evidence of women with mitochondrial mutations (Figure 14).

(6) Key Research Accomplishments:

1. The method for CAP detection of BPDE adducts is feasible, but there remains substantial problems in reducing background. One manuscript has been published.
2. We have identified a new method for radiolabeling 4ABP, using NAT2 and ^{14}C-acetyl coA. Initial results indicate that we will have both specificity and sensitivity.
3. Capillary HPLC and LIF is being developed for BPDE adducts. Preliminary data from human tissues indicates that this method is feasible, and the sensitivity and specificity has been improved. Calibration curves are now in progress to determine the limit of detection.
4. An epidemiological infrastructure has been established at LCC to continue accrual of women undergoing reduction mammoplasty.
5. Breast strains have been cultured, exposed to gamma radiation and 4-aminobiphenyl and differential p53 responses have been found. These cultures are greater than 90% epithelial. We hypothesize that women with better responses might be at reduced breast cancer risk.
6. Normal breast tissues from reduction mammoplasty donors indicates that these tissues have detectable ER alpha and ER beta, hypermethylation for BRCA1 and p16, and mitochondrial mutations.
7. Normal breast tissues from reduction mammoplasty donors express CYP1B1, CYP1A1 and Cyp3A.

(7) Reportable Outcomes

1. One manuscript has been published in the prior year.
2. Four abstracts have been submitted to the AACR and one was submitted to the Era of Hope organization.
3. Tissue repository of breast tissues from women undergoing reduction mammoplasty has been established and collection is ongoing.
4. The above data was used to support an application to the DOD for a Breast Cancer Center of Excellence.

(8) Conclusions

This grant supported the development of BPDE and 4ABP adduct detection methods, and has made substantial progress on these. The development work will continue and can then be applied to breast tissues in order to examine genotype-phenotype relationships. Thus far, we have determined the basic CAP methods for the BPDE and 4ABP, but there are problems for the former with limitations in clean-up of contaminants. As an alternate strategy, we are developing a capillary HPLC and LIF method for BPDE adducts. The preliminary data in human samples is very promising, but the quantitative accuracy still needs to be determined. We also are using an alternative radiolabeling methodology for 4ABP adducts.

The lack of completion of the adduct assays did not hinder other progress. The grant also supported the establishment and use of breast epithelial strains for examining the genotype-phenotype relationships. We have established an epidemiological study and tissue repository of women undergoing reduction mammoplasty. In addition to the samples collected while at the NCI, this a similar collection system was established at NCI. We have identified CYP expression in the breast tissues, and also have confirmed ER alpha and ER beta expression. There is a clear variability in p53 response among women, and so we hypothesize that this might be related to breast cancer risk. We also found evidence of hypermethylation of tumor suppressor genes and mitochondrial mutations in normal tissues from women who never have had cancer.

The initiation of this project was delayed at the start because Dr. Shields moved his laboratory, but the project has been fully implemented. Importantly, due to the change in institutions, less money remained available for research because of loss to indirect costs. Thus, not all tasks could be completed. Nonetheless, there has been the development of data to support several grant applications, including one for a DOD Breast Cancer Center of Excellence.

Bibliography of all Publications and Meeting Abstracts

Goldman R, Day BW, Carver T.A., Mauthe RJ, Turtletaub KW, Shields, PG. Quantitation of benzo[a]pyrene-DNA adducts by postlabeling with ^{14}C-acetic anhydride and accelerator mass spectrometry. Chemico-Biological Interactions, 2000, 126:171-183.

AACR Abstracts:

1. Enzymatic 14C-postlabeling using N-acetyltransferase as a biomarker tool: Extending the limits of detecting carcinogen-DNA adducts: Submitted by Natarajan Ganesan - Postdoctoral Fellow for 2003

2. Effects of smoking on CYP expression and PAH concentration in human lung, liver, and breast tissues: Submitted by Radoslav Goldman - Postdoctoral Fellow for 2003

3. Understanding breast cancer risk: Interindividual variability for DNA damage p53 response in human breast cells: Submitted by Ramona G. Dumitrescu - Technician for 2002

4. Analyzing promoter methylation status in normal breast tissues: Submitted by Ramona G. Dumitrescu - Technician for 2003

Era of Hope Abstract:

1. Molecular epidemiology of Breast Cancer: Development and validation of acetylation methods for carcinogen adduct detection: Submitted by Natarajan Ganesan - Postdoctoral Fellow

Personnel Receiving Pay from the Research Effort:

Peter G. Shields, M.D. - Principal Investigator

Radoslav Goldman, Ph.D. - Postdoctoral

Ramona Dumitriescu - Technician

Natarajan Ganesan, Ph.D. - Postdoctoral Fellow

Molecular Epidemiology of Breast Cancer: Development and Validation of Acetylation Methods for Carcinogen-DNA Adduct Detection

PI: Dr. Peter G. Shields, MD

Award Number: DAMD17-99-1-9309

Appendix

I. Journal Articles Published

II. Abstracts

Molecular Epidemiology of Breast Cancer: Development and Validation of Acetylation Methods for Carcinogen-DNA Adduct Detection

PI: Dr. Peter G. Shields, MD

Award Number: DAMD17-99-1-9309

I. Journal Articles Published

ELSEVIER

Chemico-Biological Interactions

Chemico-Biological Interactions 126 (2000) 171–183

www.elsevier.com/locate/chembiont

Quantitation of benzo[*a*]pyrene-DNA adducts by postlabeling with ^{14}C-acetic anhydride and accelerator mass spectrometry

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Abstract

Quantitation of carcinogen-DNA adducts provides an estimate of the biologically effective dose of a chemical carcinogen reaching the target tissue. In order to improve exposure-

Abbreviations: AMS, accelerator mass spectrometry; AP, alkaline phosphatase; BPDE, benzo[*a*]pyrene-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide; BPdG (RSRS), 7*R*,8*S*,9*R*-trihydroxy-10*S*-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene; CD, circular dichroism; dG, deoxyguanosine; DMSO, dimethylsulfoxide; LC–MS, liquid chromatography–mass spectrometry; LSC, liquid scintillation counting; MeIm, 1-methylimidazole; NP1, nuclease P1; PB, phosphate buffer; RP-HPLC, reverse phase-high performance liquid chromatography; SVPD, snake venom phosphodiesterase; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; TLC, thin layer chromatography.

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assessment and cancer risk estimates, we are developing an ultrasensitive procedure for the detection of carcinogen-DNA adducts. The method is based upon postlabeling of carcinogen-DNA adducts by acetylation with ^{14}C-acetic anhydride combined with quantitation of ^{14}C by accelerator mass spectrometry (AMS). For this purpose, adducts of benzo[*a*]pyrene-r-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BPDE) with DNA and deoxyguanosine (dG) were synthesized. The most promutagenic adduct of BPDE, 7*R*,8*S*,9*R*-trihydroxy-10S-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG), was HPLC purified and structurally characterized. Postlabeling of the BPdG adduct with acetic anhydride yielded a major product with a greater than 60% yield. The postlabeled adduct was identified by liquid chromatography–mass spectrometry as pentakis(acetyl) BPdG (AcBPdG). Postlabeling of the BPdG adduct with ^{14}C-acetic anhydride yielded a major product coeluting with an AcBPdG standard. Quantitation of the ^{14}C-postlabeled adduct by AMS promises to allow detection of attomolar amounts of adducts. The method is now being optimized and validated for use in human samples.

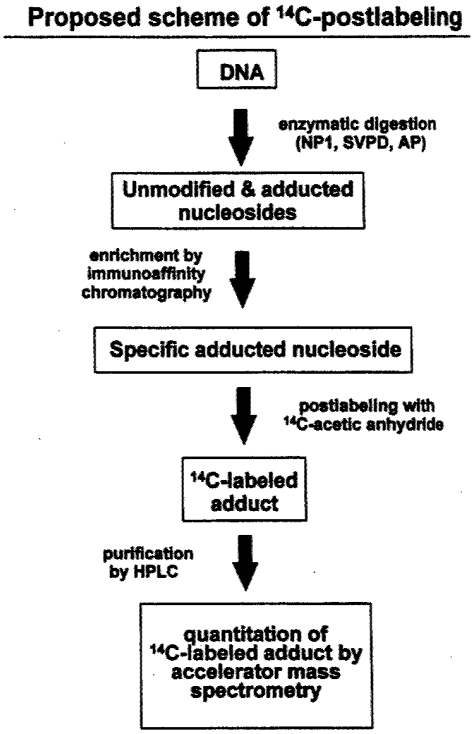
Keywords: Molecular epidemiology; Carcinogenesis ; ^{14}C-postlabeling; DNA adduct; Benzo[*a*]pyrene

1. Introduction

Epidemiological studies indicate that a substantial portion of human cancer derives from exposures to chemical carcinogens [1]. Most carcinogens form DNA adducts as a common pathway to mutagenesis [2–4]. The detection of carcinogen adducts in human tissues is a central strategy in molecular epidemiology [5] allowing: (1) estimates of the effective dose of a carcinogen in the target tissue; and (2) correlation of exposure with the incidence of disease.

Tracing the links from carcinogen exposure, DNA binding, resultant mutations and cancer contributes to an understanding of cancer etiology and the design of prevention strategies [6,7]. This task, however, is inherently difficult as humans are typically exposed to only low level complex carcinogen mixtures [8,9]. Difficulties for carcinogen-DNA adduct detection arise, in part, due to insufficient sensitivity, specificity, and reproducibility of the available analytical methods [10,11]. Detection methods based on immunoassay or ^{32}P-postlabeling may be sensitive enough [12], but lack the desired specificity unless combined with micropreparative steps (e.g. immunoaffinity chromatography) [13–15]. These additional analytical steps tend to reduce sensitivity and increase labor and/or cost beyond acceptable limits [16,17].

In our previous studies, chemical-specific measurements of benzo[*a*]pyrene-DNA adducts in human tissue reached the detectable limit in approximately 25% of examined samples [16]. This prompted us to develop a more sensitive method with retained chemical specificity for high throughput epidemiological studies. The chosen approach combines postlabeling of carcinogen-DNA adducts with [^{14}C]acetic anhydride with quantitation of ^{14}C by AMS (Scheme 1). AMS is an ultrasensitive ^{14}C detection method with documented sensitivity of zeptomole (10^{-20} mole) quantities of ^{14}C [18]. This leads to a theoretical detection limit of 1 adduct in 10^{12} to 10^{14} nucleotides based on studies with ^{14}C-labeled carcinogens [19]. This limit translates into at least 1000-fold improved sensitivity over currently



Scheme 1.

available methods [11]. We are attempting to combine this unmatched sensitivity of AMS with the versatility of the postlabeling methods. The approach is particularly promising because the postlabeling by acetylation with [^{14}C]acetic anhydride has an established chemical precedent [20]. The method should be also easily adaptable to a wide range of compounds.

The method is being developed on the most carcinogenic stereoisomer of the adduct formed by benzo[*a*]pyrene diolepoxide with deoxyguanosine which is 7*R*,8*S*,9*R*-trihydroxy-10*S*-(N^2deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene, BPdG (RSSR). Postlabeling of this widely studied precarcinogenic adduct will allow comparison with established methods. It is expected that molecular epidemiological studies based on improved detection and quantitation of DNA-adducts (and other biomarkers) will provide new, invaluable information for early detection and prevention of cancer.

2. Materials

(±)-BPDE, (+)-BPDE, (−)-BPDE, and racemic ^{3}H-BPDE were purchased from Chemsyn (Lenexa, KS). AP, DMSO, SVPD, and dG were from Sigma (St.

Louis, MO). THF/MeIm was from Perkin-Elmer/ABI (Foster City, CA). [$^{14}C_4$]acetic anhydride was from DuPont-NEN (Billerica, MA); the sealed glass ampule was kept at −20°C until experimental use. Pyridine was distilled over sodium and stored at 24°C in sealed glass ampules. DMSO was distilled under reduced pressure. All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

3. Methods

3.1. Synthesis of BPdG (RSRS)

Two methods were used to obtain sufficient quantities of standards of BPdG. Method 1B, based on derivatization of dG with BPDE, has higher yield than method 1A which relies on adduction of BPDE to DNA followed by digestion to nucleosides.

3.1.1. Method 1A

The synthesis followed a previously published method with minor modifications [20]. Calf thymus DNA (3 mg) was reacted with (+)-BPDE (0.5 mg) or racemic (±)-BPDE (0.5 mg) in 3 ml of 0.1M phosphate buffer, pH 7.5, containing 0.1 ml DMSO and 0.3 ml ethanol at 37°C for 12 h. The reaction was extracted with diethyl ether, ethyl acetate, and n-butanol (3 × 10 ml each). The DNA was precipitated from the aqueous layer with 0.3 M sodium acetate, pH 5.2, containing 2.5 volumes of ethanol. The precipitate was washed with 7:3 ethanol–water, dissolved in 0.1 M sodium acetate, pH 5.3, with 0.2 mM $ZnCl_2$, and digested with nuclease P1 (77 U/mg) for 3 h at 37°C. The buffer was adjusted with 0.3 M Na_2CO_3 to pH 8.5, and made 2 mM in $MgCl_2$. Alkaline phosphatase, 17 U/mg, and SVPD, 0.9 U/mg, were added and the reaction was further incubated for 3 h at 37°C. The digest was cleaned on a C_{18} SepPak with water (30 ml) and eluted with 3 ml of methanol. The methanolic eluent was concentrated on a Speedvac and reaction products were separated by HPLC using method 4A (see below).

3.1.2. Method 1B

The synthesis was based on a previously published protocol [21]. (+)-BPDE or racemic (±)-BPDE (0.5 mg) was dissolved in 100 μl of DMSO and reacted with dG (70 mg) in 5 ml of TFE containing 63 μl of TEA and heated to 37°C for 5 h. The solvent was evaporated on a Speedvac. The resulting mixture was resuspended in distilled water and extracted with n-butanol (4 × 10 ml). The organic layers were pooled, washed with water (3 × 50 ml), and concentrated on a rotary evaporator. The residue was dissolved in methanol and separated by HPLC using method 4A (see below).

3.2. Acetylation of BPdG with acetic anhydride

The yield of acetylation was compared in two solvent systems. The reaction had a high yield in both pyridine and THF/MeIm, but the second method is faster and more efficient at low concentrations of starting material.

3.2.1. Method 2A

BPdG (RSRS) (10 nmol) was acetylated for 2 h at 35°C with acetic anhydride (3 μl) in 20 μl of anhydrous pyridine. The reaction was terminated by the addition of 20 μl of 50% aqueous methanol and evaporation of the solvents to dryness on a Speedvac. The product of the reaction was purified by HPLC method 4B (see below). The separated peaks were collected and analyzed by LC-MS.

3.2.2. Method 2B

Acetic anhydride (3 μl) was dissolved in 400 μl of THF/MeIm (84% THF, 16% MeIm). An aliquot of 20 μl was added to HPLC-purified BPdG (1-100 pmol). The reaction was carried out for 20 min at 22°C, stopped with 20 μl of 50% aqueous methanol, and evaporated to dryness on a Speedvac. The reaction products were analyzed by HPLC method 4B and the major product was further analyzed by LC-MS.

3.3. Acetylation of BPdG with [$^{14}C_4$]acetic anhydride

3.3.1. Method 3A

BPdG (RSRS) (2 nmol) was acetylated for 2 h at 35 °C with 8.4 mCi/mmol [$^{14}C_4$]acetic anhydride (3 μl) in 12 μl of anhydrous pyridine. The reaction was terminated by evaporation of the solvent on a Speedvac. The concentrated reaction mixture was dissolved in 200 μl of methanol and evaporated to dryness three times. The product of the reaction was purified by HPLC method 4C (see below). The peak corresponding to pentakis(acetyl) BPdG was collected and the column was washed with 200 ml of THF, 200 ml of water, and 200 ml of methanol. Aliquots of the purified [^{14}C]AcBPdG were reinjected on the washed HPLC column. The fractions co-eluting with AcBPdG standard were analyzed by AMS for ^{14}C-content (see below).

3.3.2. Method 3B

[$^{14}C_4$]acetic anhydride (3 μl) was dissolved in 400 μl of THF/MeIm (84% THF, 16% MeIm). A 20 μl aliquot was added to the HPLC-purified RSRS stereoisomer of BPdG (10 pmol) under a controlled anhydrous atmosphere in a polycarbonate reaction chamber (Coy Laboratories, Grass Lake, MI). The reaction was carried out for 20 min at 22°C, stopped by addition of 50% aqueous CH_3OH (20 μl), and evaporated to dryness on a Speedvac. The reaction products were loaded on a Waters C18 SepPak cartridge (100 mg) together with 5 pmol of AcBPdG standard. The SepPak was washed with 0.03% aqueous CH_3CO_2H (50 ml), 10% aqueous methanol (50 ml), 30% aqueous methanol (50 ml), and 20 mM ammonium acetate,

pH 7.4 (50 ml). [^{14}C]AcBPdG was eluted with methanol (2 ml). Aliquots of the methanolic eluent were analyzed by HPLC, method 4C, and the ^{14}C content of the HPLC fractions was measured by AMS (see below).

3.4. Accelerator mass spectrometry

Measurement of the radiocarbon content of the samples was carried out using accelerator mass spectrometry (AMS) [18,22]. AMS measures the amount of radioisotope, e.g. ^{14}C relative to a stable isotope of the same element. In this study, ^{14}C was measured relative to ^{13}C and then normalized to the ratio of ^{14}C/^{12}C using the Australian National University sugar standard [23] as reference. For AMS analysis, HPLC samples were converted to graphite by a two step process involving combustion of the samples to CO_2 followed by reduction to filamentous graphite, as described previously [24]. Because the complete process to graphite works best with 1–2 mg of total carbon, and to insure a well-known and constant amount of carbon in each HPLC fraction, 2 mg of tributyrin (providing ca. 1 mg of carbon), well characterized with respect to radiocarbon content, was added to fractions as a carrier carbon prior to combustion. The samples were measured as graphite and converted to amol radiocarbon/mg sample by subtracting carbon-14 contribution from control material and carrier carbon [18,24].

3.5. Electrospray mass spectrometry

Mass spectrometric determinations were carried out on Perkin-Elmer/Sciex API 1 spectrometer equipped with an atmospheric pressure ionization source and an IonSpray interface which was maintained at 5 kV. The orifice was maintained at 70 V, high purity N_2 flowing at 0.6 L/min served as curtain gas, and high-purity air maintained at 40 psi was used for nebulization. Samples were introduced using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a diode array 1040A detector.

The analytes (BPdG or AcBPdG) were introduced into the mass spectrometer without column separation and without splitting of the effluent. As little as 2 pmol of analyte was injected into the ionization source in a 40 μl/min flowing stream of 50% aqueous acetonitrile containing 0.05% TFA. Mass spectra were acquired every 6-12 s over the range of m/z 300 to 1000 (m/z 0.1 resolution). Analytes were detected as their $[M+H]^+$, $[M+K]^+$, or $[M+Na]^+$ions.

3.6. HPLC separation of adducts

Analytes were injected with a Waters 717Plus autosampler into a Hewlett-Packard 1050 Series liquid chromatograph equipped with a diode array 1040A detector.

3.6.1. Method 4A

The separation of BPdG was achieved with a C_{18} column (Beckman Ultrasphere ODS, 5 μm particle size, 10 × 150 mm) using a gradient of methanol (solvent

A)–water (solvent B) (0–3 min, 40% A; 3–5 min, linear gradient to 50% A; 5–22 min, linear gradient to 55% A, 22–26 min, linear gradient to 99% A; 26–30 min isocratic at 99% A) as the mobile phase at a flow rate of 3 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of BPdG (RSRS) was 18 min.

3.6.2. Method 4B

Acetylated BPdG was separated from other analytes on a C_{18} column (Beckman Ultrasphere ODS, 5 μm particle size, 4.6 × 250 mm) using a gradient of methanol (solvent A)-water (solvent B) (0–20 min, 70% A; 20–22 min, linear gradient to 90% A; 22–29 min, isocratic at 90% A; 29–30 min, linear gradient to 100% A; 30–35 min, isocratic at 100% A) as the mobile phase at a flow rate of 1 ml/min with UV monitoring at 279 and 344 nm. Under these conditions the retention time of pentakis(acetyl) BPdG (RSRS) was 22.9 min.

3.6.3. Method 4C

A longer gradient was used for cleanup of ^{14}C-acetylated BPdG using the Beckman Ultrasphere ODS column, 5 μm particle size, 4.6 × 250 mm. The gradient was as follows : methanol (solvent A)-water (solvent B) (0–10 min, 20% A; 10–13 min, linear gradient to 50% A; 13–25 min isocratic at 50% A; 25–28 min, linear gradient to 70% A; 28–38 min, isocratic at 70% A; 38–40 min, linear gradient to 90% A; 40–50 min, isocratic at 90% A; 50–53 min, linear gradient to 100% A; 53-60 min, isocratic at 100% A). The retention time of pentakis(acetyl) BPdG (RSRS) was 44.5 min under these conditions. Fractions of 1 ml were typically collected and further analyzed for ^{14}C-content by AMS as described above.

3.7. UV-Vis and CD spectra

All spectrophotometric measurements were carried out using a Beckman DU640 spectrophotometer. The concentration of BPdG was measured at 279 nm using $\varepsilon = 40\,984\ M^{-1}\ cm^{-1}$. CD spectra were recorded on a JASCO J-500A spectropolarimeter.

4. Results

Derivatization of DNA with (+)-BPDE or (±)-BPDE yielded one major product as previously described [20]. The product was purified from the enzymatic DNA digest by HPLC (Method 4A) and characterized. CD spectropolarimetry showed that the product was the RSRS stereoisomer of BPdG [20] (Fig. 1A). The mass spectrum of the purified adduct was consistent with the mass of a BPDE adduct with dG (BPdG) (Fig. 1B). Synthesis of BPdG from dG and (±)-BPDE was carried out in TFE/TEA [21]. The reaction afforded a product with the same spectral characteristics as the BPdG adduct isolated from BPDE-derivatized DNA (data not shown), but in higher yield. This method was used for the preparation of

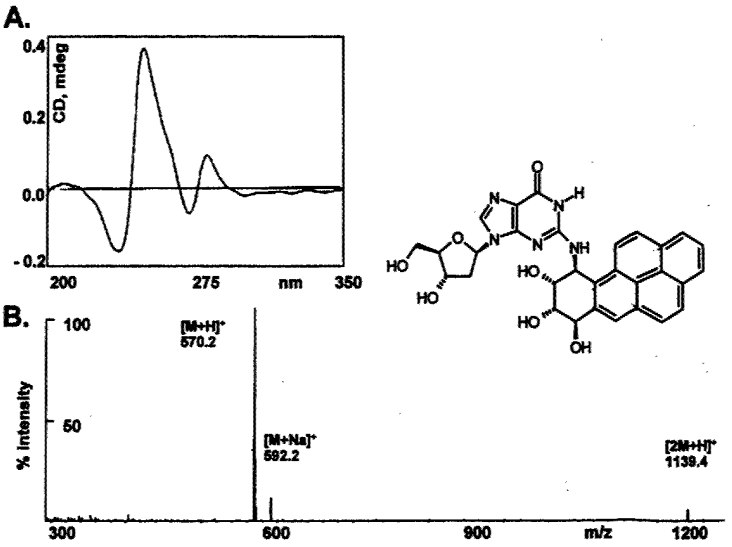


Fig. 1. Characterization of the major BPdG stereoisomer. (A) CD spectra of the HPLC-purified BPdG (RSRS) are identical with previously published CD spectra. (B) The quasimolecular ion determined by electrospray mass spectrometry is consistent with that of the BPdG adduct.

standards of the BPdG adducts. The adducts were used for the optimization of postlabeling reactions and as chromatographic standards.

Postlabeling of the BPdG (RSRS) adduct by acetylation with acetic anhydride was initially carried out as described previously [20]. Acetylation in pyridine yielded one major product in >60 % yield. The product was isolated by HPLC (Method 4B) (Fig. 2A). Its protonated quasimolecular ion (m/z 779; electrospray MS) of the major product was consistent with addition of five acetyl groups to BPdG (RSRS), i.e. pentakis(acetyl) BPdG (AcBPdG) (Fig. 2B). The AcBPdG was further used as a standard for HPLC separations of ^{14}C-AcBPdG.

Use of THF/MeIm as a solvent gave higher postlabeling yields when picomolar amounts of the BPdG adduct were acetylated [25]. Pentakis(Acetyl) BPdG was also the major product under these conditions as verified by LC-MS, but the reaction was faster and more efficient (data not shown).

Acetylation of BPdG (RSRS) at the nanomolar scale with excess [$^{14}C_4$]acetic anhydride yielded a major peak in the accelerator mass spectrometric (AMS) profile of the HPLC fractions (Fig. 3A). This radiolabeled product co-eluted with the AcBPdG standard. Aliquots of the isolated [^{14}C]AcBPdG product were further analyzed by AMS after extensive washing of the column to remove background ^{14}C (i.e. the unidentified ^{14}C-species retained on the HPLC column). The AMS analysis of samples obtained by serial dilution of the [^{14}C]AcBPdG using the washed column was carried out according to established procedures [18,24]. The AMS analysis fractions coeluting with the AcBPdG standard allowed construction of a theoretical dose-response curve for detection of the postlabeled adduct (Fig. 3B). The detection

limit under these conditions was 1 fmol of adduct, even though the fractions showed a 10-fold higher level of background ^{14}C than expected (Fig. 3B).

An additional cleanup step with a C_{18} SepPak cartridge preceding the HPLC separation eliminated the background ^{14}C. Postlabeling of picomolar amounts of BPdG (method 3B) combined with SepPak cleanup and HPLC separation yielded AMS profiles with the major peak co-eluting with the AcBPdG standard (Fig. 4A). Analyses of aliquots of this reaction allowed construction of a new dose-response curve for detection of the postlabeled [^{14}C]AcBPdG (Fig. 4B). The detection reached the theoretical limit of 100 attomoles of adduct using these conditions.

5. Discussion

Cancer results from an as yet unidentified interplay of environmental/endogenous exposures and genetic susceptibilities [5,6]. Many ongoing studies in our laboratory [26] and elsewhere [27,28] examine the contribution of genetic polymorphisms to the variability of function in both 'caretaker' (metabolism, detoxification, DNA repair) and 'gatekeeper' (cell cycle control, apoptosis) cancer susceptibilty genes [29]. Carcinogen-DNA adducts serve as biomarkers that link genetic susceptibility with an exposure [10]. For example, in lung [30], bladder [31,32], and other tissues [33],

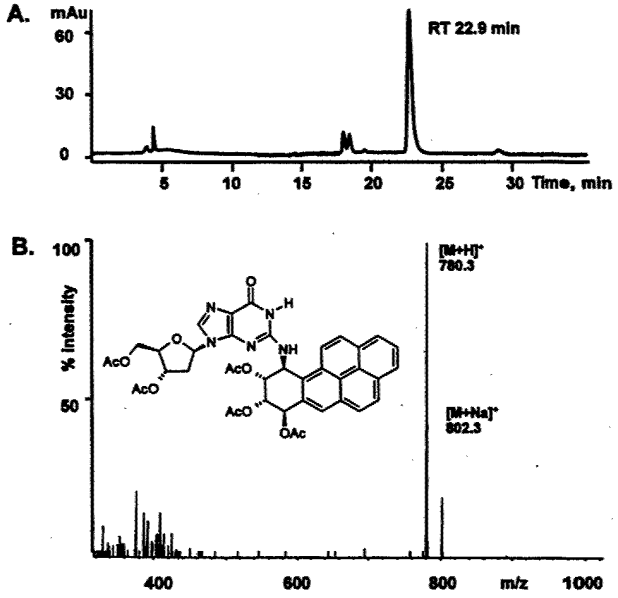


Fig. 2. Characterization of the BPdG postlabeled by acetylation (AcBPdG). (A) The HPLC profile (monitored at 344 nm) shows one major product with a retention time of 22.9 min (Method 4B) (B) The mass spectrum of the HPLC purified peak (22.9 min) is consistent with the pentakis(acetyl) BPdG adduct.

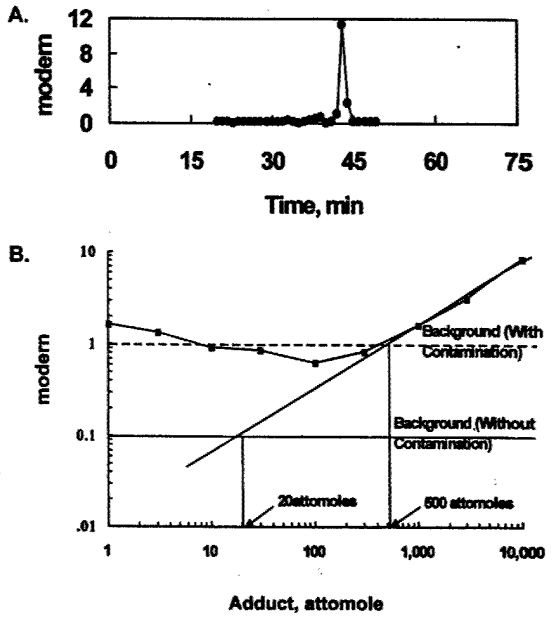


Fig. 3. Analysis of the ^{14}C-acetylated BPdG (nanomolar scale). (A) HPLC profile of the [^{14}C]AcBPdG with AMS detection (Method 4C). The major ^{14}C-peak, RT 44.2 min, coelutes with the synthesized AcBPdG standard. Modern is defined as 5.9×10^{10} $^{14}C/g$ of carbon (approximately the concentration of ^{14}C in the atmosphere in 1950 [23]). (B) Dose-response of [^{14}C]AcBPdG aliquots of the major peak. The graph (log/log scale) shows that the background in the samples is about ten-fold higher than the background ^{14}C in the tributyrin solvent. The squares represent individual measured values. The straight line is an extrapolation to background without contamination.

DNA adduct levels are higher in persons with hypothesized 'at risk' genetic variants. This shows that the carcinogen-DNA adducts estimate the total burden of a particular exposure in the target tissue and suggest a link to cancer risk [30,34]. The chemical specificity and sensitivity of the DNA adduct-detection are two key issues limiting the application of most currently available methods to large epidemiological datasets. The ^{14}C-postlabeling is an alternative method with the potential to be both specific and sensitive. The procedure, is being developed using the well known carcinogen benzo[*a*]pyrene which is related to important environmental and occupational exposures including smoking.

The acetylation reaction yielded one major product, pentakis(acetyl) BPdG (AcBPdG), in high yield. Postlabeling by acetylation with [^{14}C]acetic anhydride was analogous to the labeling with acetic anhydride as expected. The major peak of ^{14}C detected by AMS co-eluted with the AcBPdG standard in the HPLC profile (Fig. 3A and Fig. 4A). The area under the peak was proportional to the amount of postlabeled adduct injected on the column (Fig. 4B). It is important to separate the excess ^{14}C from the ^{14}C-postlabeled adduct prior to the HPLC analyses (Fig. 3B). Impractical amounts of solvent (>500 ml) had to be used for column washing when >100 dpm of ^{14}C was applied to the column. The cleanup was still incom-

plete after the wash with 600 ml of solvents when $> 10^6$ dpm of ^{14}C was loaded on the column (Fig. 3B). The excess ^{14}C-label was therefore washed out on a C_{18} SepPak cartridge prior to the HPLC analysis (Fig. 4). The procedure provided sufficient cleanup without affecting recovery. The recovery tested with synthesized ^{3}H-AcBPdG was always greater than 90% (data not shown). The current limit of detection in the attomolar range promises excellent sensitivity for the detection of adducts in human DNA, provided that the reactions/cleanup proceed without additional complications at a smaller scale. Optimization of the washing step and of the reaction at femtomolar and attomolar starting amounts of adduct are underway.

This report summarizes the development of a novel postlabeling method based on acetylation with ^{14}C combined with AMS quantitation. The presented preliminary results are encouraging. The HPLC separation of postlabeled adducts provides chemical specificity which will be further increased when combined with immunoaffinity chromatography for isolation of the adduct of interest from DNA. The assay also promises excellent sensitivity. The quantitation of the ^{14}C by AMS does not rely on radioactive but rather determines the ratio of ^{14}C to ^{12}C. This provides a significant increase in sensitivity compared to the short-lived ^{32}P isotope used in classical postlabeling. The projected sensitivity should allow detection of adducts in the range of 1 adduct in 10^{12} nucleotides. We should stress that this report is preliminary, that the optimization and validation steps will be crucial to prove the utility of this method, and that the ultimate test will be the analysis of human samples.

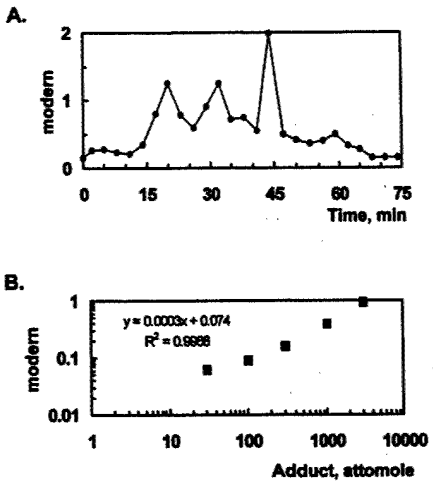


Fig. 4. Analysis of the ^{14}C-acetylated BPdG (picomolar scale). (A) HPLC profile of the [^{14}C]AcBPdG with AMS detection after preparatory cleanup on a C18 seppak cartridge (Method 4C). The major ^{14}C-peak (44.2 min) coelutes with the synthesized AcBPdG standard. Dose–response of ^{14}C-AcBPdG aliquots of the major peak. The background in the samples is similar to the background ^{14}C in the tributyrin solvent, i.e. about 0.1 Modern. The projected sensitivity is about 100 amol of adduct.

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Molecular Epidemiology of Breast Cancer: Development and Validation of Acetylation Methods for Carcinogen-DNA Adduct Detection

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I. Abstracts

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Enzymatic 14C-postlabeling using N-acetyltransferase as a biomarker tool: Extending the limits of detecting carcinogen-DNA adducts

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Quantification of low levels of irreversible DNA damage (carcinogen-DNA adducts) is important in selectively identifying individuals as high risk cancer subjects. As a prototype we are establishing a new assay for the detection of 4-aminobiphenyl-DNA adducts in human samples and validate the same to prove its utility in carrying out epidemiological studies. The novelty of this assay is based on a modified 14C postlabelling method using the enzyme N-acetyltransferase (NAT) (E.C.-2.3.1.5) with labeled acetyl-CoA as the 14C donor. 4-ABP-DNA standards were prepared by reacting N-hydroxy-4-aminobiphenyl (synthesized from 4-Nitrobiphenyl) with calf thymus DNA. The prepared 4-ABP-DNA standard was subjected to an alkaline hydrolysis procedure, releasing 4-ABP from DNA. After extraction into hexane and HPLC purification, the released 4-aminobiphenyl (confirmed by mass spectrometry) was subjected to enzymatic acetylation using 3H Acetyl CoA as the acetyl donor. The reaction mix was subjected to a single step clean-up procedure using a C18 SEPAK column. The reaction products were collected in methanol, dried and redissolved in water/acetonitrile mix for analysis by HPLC. Unreacted acetyl-CoA eluted out at the solvent front while the acetylated product eluted before the starting material during the separation. The 3H label was distributed between acetyl CoA and the acetylated product without spillover into other byproducts. For very low levels of adduction, the samples would be subjected to an additional purification step involving immunoaffinity chroamtography. The acetylated product will be detected and quantified using accelerator mass spectrometry (in collaboration with Turteltaub KW, CA). Following the documented sensitivity of AMS procedure, we expect a thousand-fold increase in adduct detection sensitivity over the existing procedures.

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Effects of smoking on CYP expression and PAH concentration in human lung, liver, and breast tissues

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The effect of smoking on expression of CYP1A1, CYP1B1, and CYP3A and activation of eleven polycyclic aromatic hydrocarbons (PAH) was analyzed in 95 tissue samples from 50 individuals. The tissues are from autopsies of cancer free subjects with known smoking status of the individuals and concentration of the PAH compounds. Our previous analyses showed that smoking increases concentration of PAH in the lung tissue. Expression of the CYPs was evaluated by immunohistochemistry. CYP1A1 was detected in 38 of 50 lung and 13 of 16 breast samples, but none of 27 liver samples. CYP1B1 was present in 45 of 50 lung, 14 of 16 breast and 3 of 27 liver samples. CYP3A was detected in 44 of 50 lung, 8 of 16 breast and all 27 liver samples. In the lung of smokers, the expression of CYP1A1 ($p<0.0001$), CYP1B1 ($p=0.004$) and CYP3A ($p=0.005$) was significantly elevated in the TypeII cells. CYP1A1 was also elevated in bronchial cells by smoking ($p=0.006$) but smoking did not increase the expression of any CYP in Type I cells. The expression of the three CYPs in the breast was not correlated with smoking status. There was little correlation in expression of the enzymes in matched lung and breast tissues of the same individual. A preliminary analysis shows that CYP1A1 induction by smoking does not vary with race and gender; but the induction of CYP1B1 in the lung was detected in Caucasian subjects ($n=17$; $p=0.01$) but not African American subjects ($n=27$; $p=0.26$). This is due to positive CYP1B1 staining in lung tissue of African American non-smokers. The study shows clearly induction of PAH activating CYPs in lung tissue of smokers. The breast tissue expresses the carcinogen activating CYPs but smoking does not induce expression. The preliminary results indicate some differences based on race of the individuals.

Analyzing promoter methylation status in normal breast tissues

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Introduction: DNA hypermethylation of gene promoter CpG islands has been described in almost every tumor type. Familial cancer genes as well as other genes can be silenced by promoter hypermethylation. This epigenetic change inactivates genes involved in cell cycle (p16), DNA repair (BRCA1), matrix metalloproteinase activity (TIMP3), estrogens signaling (ER) and FHIT(fragile histidine triad), a putative tumor suppressor gene.

Purpose: To identify intermediate markers of breast cancer risk.

Hypothesis: Detection of hypermethylation in normal tissues from healthy patients may be a marker for altered one carbon metabolism

Methods: Human breast tissues were collected from normal donors who underwent reduction mammoplasty surgery. DNA was isolated from frozen tissues. Bisulfite modification was performed for 16h at 50°C. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines(but not methylated cyotsines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving differences between methylated and unmethylated DNA. The methylation status of BRCA1, ER, TIMP3 and FHIT was determined by the method of Methylation specific PCR. The improved MSP, which incorporated a nested, two-stage PCR approach and is more sensitive(1 methylated allele in >50,000 unmethylated alleles) was used for detecting p16 methylation. Specific primers were used to selectively amplify unmethylated and methylated alleles.

Results: The hypermethylation was detected in 5 out of 9 human breast samples of cancer-free patients for p16, 4 out of 9 for BRCA1, 3 out of 9 for ERα and no aberrant methylation was detected for TIMP3 and FHIT. The unmethylated alleles for these genes were found in all human breast samples. Alternative methods will be used to confirm these results.

Conclusions: Our studies suggest that these genes hypermethylation status may be a potential biomarker for an early detection of noninherited breast cancer, by identifying those patients at higher risk. These findings will be tested in a larger number of human samples in order to evaluate a potential target for breast cancer prevention.

Understanding breast cancer risk: Interindividual variability for DNA damage p53 response in human breast cells

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Introduction: The genetic determinants for sporadic breast cancers remain largely undefined. Prior research has focused mostly on carcinogen metabolizing and DNA repair genes. Other genetic traits need to be defined that affect breast carcinogenesis. p53 protects mammals from neoplasia by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of stresses.

Purpose: To determine the interindividual variation for p53 induction and check point control in response to DNA damage using human mammary epithelial cells.

Methods: The samples from normal donors who underwent reduction mammoplasty were used to set up primary cell cultures. Cells were used for experiments when they reached confluency (in 3-5 weeks), following first, second or fourth passage. Staining of cells for senescence-associated beta-galactosidase was performed for cells in passage 4. Cytokeratin staining of cells by immmunofluorescence was used to determine the percent of contamination by fibroblasts in human mammary epithelial cell cultures. Cells were irradiated at 5Gy, 10Gy and 20Gy in order to generate a dose response curve. The incorporation of bromodeoxyuridine (BrdU) was used to indicate the proliferative index.

Results: Cytokeratin staining showed that the cultures expressed 80-99% epithelial cells. We observed a differential amount of p53 induction among the strains at different doses of gamma radiation. The radiation exposures are resulted in a dose-dependent increase in p53 expression from 5Gy to 20Gy and a leveling off in p53 induction at 20Gy. A range from 2-fold to 5-fold increase in p53 was demonstrated at the highest level of radiation (20Gy). Based on our results the strains can be separated into 3 groups: low, medium and high responders. The BrdU incorporation assay showed that even at the lowest dosage (5 Gy) most of the cells arrested in G1/S phase.

Conclusions: These results indicate an interindividual variation in DNA damage p53 response.This may affect breast cancer risk because of the way breast tissue respond to DNA damage. Human mammary epithelial cells response to DNA damage by arresting their cell cycle in G1/S phase.

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Molecular epidemiology of Breast Cancer: Development and validation of acetylation methods for carcinogen adduct detection

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Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. The study of carcinogen-DNA adducts can provide corroborative evidence for the importance of genetic susceptibilities in breast cancer risk. We are establishing new assays for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies, and determine adduct levels in relation to metabolizing gene polymorphisms. Two assays are under development, one for benzo(a)pyrene (BP) and the other for 4-aminobiphenyl (4ABP). The first is based on capillary HPLC and laser-induced fluorescence. This method, due to the substantially improved technology, is more sensitive and easier to do compared to earlier methods. We have been able to detect the adducts in human samples and are now validating the method. The 4ABP assay is highly novel because it is an enzymatic radiolabeling method that uses commercially available N-acetyl-transferase, so that we have high specificity. The adducts are labeled with ^{14}C acetyl CoA and then the level of radioactivity is quantitated by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). With all these, we will apply the methods to surgical breast tissues and relate these different parameters as they relate to age, gender, race, and smoking. We have breast tissues from 235 donors (200 women, 35 men), and have established cell strains from 40 of them. These studies can then lead to the study of the genetic bases for these phenotypes. Thus, this study is important because it holds the promise of identifying several new genetic traits for breast cancer risk.